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The Effects of Multiple Abiotic Stressors on the Susceptibility of the Seagrass Thalassia Testudinum to Labyrinthula sp., the Causative Agent of Wasting Disease

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THE EFFECTS OF MULTIPLE ABIOTIC STRESSORS ON THE SUSCEPTIBILITY OF THE SEAGRASS *THALASSIA TESTUDINUM* TO *LABYRINTHULA* SP., THE CAUSATIVE AGENT OF WASTING DISEASE

By

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Dedication

In memory of my grandmother, Iola Bishop (1912-2002), who always believed "education was something that could never be taken away"

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Abstract

In the past century, seagrasses have experienced massive die off episodes in what has been collectively referred to as "wasting disease." Researchers generally agree that wasting disease is caused by a protist of the *Labyrinthula* genus, and that environmental stressors can make some populations of seagrasses more susceptible to infection. The purpose of this study was to examine the combined effects of elevated salinity, elevated temperature, elevated sulfide and night-time hypoxia on *Thalassia testudinum* health and its response to *Labyrinthula* sp. infection under controlled conditions. To test these effects, microcosms were utilized and individual seagrass shoots were randomly assigned to treatment groups consisting of various combinations of abiotic stressors. They were then infected with *Labyrinthula* sp. and monitored for lesion formation and a reduction in photosynthetic efficiency. It was hypothesized that seagrasses incubated under the application of a given stressor would show evidence of declining health, and, in turn, would be more prone to infection, as quantified by lesion size and reduced photosynthetic capacity. Results indicated that abiotic stressors have little effect on *T. testudium's* ability to resist infection from *Labyrinthula* sp. However, the *Labyrinthula* sp. was highly sensitive to abiotic stressors, specifically salinity, indicating that the health of the pathogen greatly contributed to the severity of the disease. Therefore, the stress thresholds of both the host seagrass and the pathogen need to be considered. Indeed, the interaction(s) among *T. testudinum*, *Labyrinthula* spp. and the environment are complex and not as linear as previously thought.

1

Introduction

1.1 Evolution, Taxonomy, Distribution and Adaptations

Seagrasses are a paraphyletic group of marine angiosperms found in both temperate and tropical waters worldwide. Compared with the number of terrestrial angiosperms (~250,000 species), seagrasses exhibit low taxonomic diversity (Orth *et al.* 2006). There are approximately only 60 species and all inhabit relatively shallow estuarine and coastal waters (Papenbrock 2012). Seagrasses are not a single taxonomic group; three lineages of terrestrial plants secondarily colonized marine habitats approximately 70 million to 100 million years (Les *et al.* 1997; Orth *et al.* 2006; Papenbrock 2012). Currently, seagrasses are considered an ecological group comprised of four families (den Hartog and Kuo 2006; Papenbrock 2012).

Seagrasses are believed to have independently evolved adaptations to the marine environment at least three to five times during their re-colonization of the sea (Papenbrock 2012). These adaptations are mainly related to submarine light acquisition, osmoregulation in a saline environment and hydrophilous reproduction. For example, the sexual reproduction of these marine angiosperms involves submarine flowering and pollination with specialized pollen and seed dispersal (den Hartog 1970; Les *et al.* 1997; Orth *et al.* 2006; Papenbrock 2012).

Seagrasses require large amounts of light and have developed many adaptations to increase their light acquisition. Sediments in which seagrasses grow are highly organic (Hemminga and Duarte 2000; Koch and Erskine 2001; Terradoes *et al.* 1999); microbial

activity in these sediments results in hypoxic conditions and an accumulation of toxic hydrogen sulfide (Carlson *et al.* 2002; Dawes *et al.* 2004; Erskine and Koch 2000; Koch and Erskine 2001). Oxygen, from photosynthesis, is used to oxidize hydrogen sulfide into non-toxic compounds and to oxygenate the rhizosphere (Carlson *et al.* 2004). Additionally, seagrasses possess aerenchyma, or air pockets in their leaf blades, that make the plant buoyant and ensure that the leaves receive the most light possible for photosynthesis. The aerenchyma are also integral for internal gas transport (den Hartog 1970; Les *et al.* 1997; Orth *et al.* 2006). During light periods, oxygen is stored in the aerenchyma and transported to the roots and rhizomes for sediment oxidation (Papenbrock 2012). This extensive network of roots and rhizomes is not only important for anchoring the plant, but is also essential in maintaining soil chemistry (Orth *et al.* 2006; Terrados *et al.* 1999). Epidermal chloroplasts, a reduced leaf cuticle and an absence of stomata also contribute to improved chemical transport and better photosynthetic efficiency (den Hartog 1970; Les *et al.* 1997; Orth *et al.* 2006; Papenbrock 2012).

Seagrass osmoregulation adaptations allow them to inhabit saline waters that are in constant flux. Seagrass tissues are hyperosmotic relative to their environment (Touchette 2007). To generate and maintain high osmotic pressure, seagrasses utilize selective ion flux. The plasma membrane of epidermal cells in the leaf blades and sheath tissues are invaginated, increasing the surface area for selective carriers and channels, thus giving seagrasses greater control of their solute flux (Dawes et al. 2004; Papenbrock 2012). Vacuolar ion sequestering and cytosolic osmolyte accumulation are also employed by seagrasses for osmoregulation. Toxic ions, such as Na^+, are isolated in

vacuoles away from important metabolic processes. Other ions, such as K^+, are important seagrass osmolytes. Some seagrasses, such as *Zostera marina*, possess highly selective K^+ channels to maintain solute flux (Fernandez *et al.* 1999; Touchette 2007). When needed, seagrasses are capable of breaking down low molecular weight organic solutes, such as organic acids, carbohydrates and free amino acids, to utilize as osmoprotectants (Touchette 2007).

1.2 Ecological and Economical Importance of Seagrass Beds

Despite relatively low species richness, seagrass meadows are highly productive and form one of the most important marine habitat types, both ecologically and economically (Dawes *et al.* 2004; den Hartog 1970; Duarte 2001, 2002; Orth *et al.* 2006; Short *et al.* 2000). Economically, seagrass meadows are responsible for supporting a multi-million dollar recreational and commercial fishing and boating industry (Dawes *et al.* 2004; Milon and Thunberg 1993; Thomas and Stratis 2001; Virnstein 1999; Virnstein and Morris 1996; Wingrove 1999). In Florida alone, a major portion of the economy relies heavily upon the health of seagrass beds, and includes tourism and the marine aquarium industry (Dawes *et al.* 2004).

Seagrass meadows are also vital components of many ecosystems. The multitude of ecological services seagrasses provide are invaluable and are a result of their ability to influence their biological, physical and chemical surroundings (Orth *et al.* 2006; Papenbrock 2012). Seagrass meadows serve as submarine pastures for grazers such as green sea turtles, dugongs and manatees (Orth *et al.* 2006). They are also the foundation to many trophic systems and support a multitude of diverse consumers (Duarte 2002;

Hemminga and Duarte 2000; Koch *et al.* 2007b; Orth *et al.* 2006). Seagrasses are important basal organisms for detrital food webs (Fonseca and Fisher 1986; Lubber *et al.* 1990; Mazzotti *et al.* 2007) and vital components of benthic and epibenthic communities (Dawes *et al.* 2004; Short *et al.* 2000).

Seagrass beds provide habitat to a diverse assemblage of vertebrates, invertebrates and microbial organisms (Duarte 2002; Hemminga and Duarte 2000; Orth *et al.* 2006), including benthic and pelagic species (Fonseca and Fisher 1986; Lubber *et al.* 1990; Mazzotti *et al.* 2007). Florida Bay alone has over 100 fish and 30 crustacean species that utilize seagrass habitat. Indeed, all commercially and economically important species in Florida depend on seagrass meadows at some point in their lives (Dawes *et al.* 2004; Ogden 1980; Thayer and Ustach 1981; Thayer *et al.* 1978, 1984). Seagrasses not only provide shelter for commercially and recreationally important species (Beck *et al.* 2001; Orth *et al.* 2006), but also provide essential habitat for many endangered species (Duarte 2002). Seagrass beds even serve as critical habitat to many avian species (Dawes *et al.* 2004; Kenworthy *et al.* 1988b; Livingston 1990; Stedman and Hanson 1997; Thayer *et al.* 1997; Valentine *et al.* 1997). In addition to their role as habitat for mature organisms, seagrass meadows serve as breeding and nursery grounds for a multitude of vertebrates and invertebrates, including many economically important fish and shellfish species (Beck *et al.* 2001; Fonseca and Fisher 1986; Heck *et al.* 2003; Lubber *et al.* 1990; Mazzotti *et al.* 2007; Orth *et al.* 2006; Papenbrock 2012; Robblee *et al.* 1991).

Seagrasses are also important in that they modify their physical environment. The subterranean roots and rhizomes stabilize sediment and prevent resuspension, thus improving water quality and clarity (Dawes *et al.* 2004; Duarte 2002; Fonseca and Fisher

1986; Hemiminga and Duarte 2000; Kenworthy *et al.* 1988b; Koch *et al.* 2007b; Livingston 1990; Lubber *et al.* 1990; Mazzotti *et al.* 2007; Orth *et al.* 2006; Thayer *et al.* 1997; Stedman and Hanson 1997; Valentine *et al.* 1997;). Above ground structures prevent erosion of shorelines by reducing wave action and modifying currents (Duarte 2002; Hemminga and Duarte 2000; Orth *et al.* 2006). Their ability to trap and filter contaminants also improves water quality (Dawes *et al.* 2004; Short *et al.* 2000).

Additional roles of seagrass meadows include mitigating chemical processes such as primary production, nutrient cycling and oxygen production. Seagrasses are highly productive primary producers (Dawes *et al.* 2004; Duarte 2002; Short *et al.* 2000). The annual net production of seagrass beds worldwide is estimated to be approximately 6.0×10^{14} gC yr^{-1} (Duarte 2002; Duarte and Chiscano 1999). Not only are seagrasses themselves highly productive, but the community of epiphytic algae that reside on seagrass blades are also important primary producers (Duarte and Chiscano 1999; Orth *et al.* 2006). Carbon sequestered from the atmosphere by seagrass meadows is an important source of carbon for the detrital pool and contributes to nutrient limited deep sea organic matter (Duarte 2002; Duarte *et al.* 2005; Orth *et al.* 2006). Seagrass beds are also important in the trapping, cycling and retention of nutrients (Dawes *et al.* 2004; Duarte 2002; Hemminga and Duarte 2000; Koch *et al.* 2007b; Orth *et al.* 2006; Short *et al.* 2000). Finally, seagrasses oxygenate the surrounding waters and sediment, allowing diverse faunal and infaunal assemblages (Duarte 2002; Short *et al.* 2000).

Seagrass beds not only influence their immediate geographical surroundings, but have also been shown to contribute to outlying ecosystems. Ecologically, they serve as important links for estuarine, marine, coastal, and even terrestrial environments (Duarte

2002; Mazzotti *et al.* 2007; Orth *et al.* 2006). Adjacent ecosystems rely upon seagrasses for organic carbon export (Duarte 2002). Many mammals and avian species, ranging from fully aquatic to terrestrial, depend on organisms whose trophic foundations are seagrass beds (Duarte 2002; Touchette 2007). When compared to other habitats, both marine and terrestrial, the services provided by seagrass meadows are extremely valuable, both ecologically and economically (Orth *et al.* 2006).

1.3 Seagrass Decline

Unfortunately, seagrass populations are declining worldwide in both temperate and tropical regions (Bergmann *et al.* 2010; Bull *et al.* 2012; Duarte 2002; Papenbrock 2012; Orth *et al.* 2006; Touchette 2007; Waycott *et al.* 2009). The causes, often debated, are attributed to biotic and abiotic stressors, human and natural antagonists and range from a global to a local scale.

Anthropogenic influences, either directly or indirectly, are almost unanimously attributed as the major contributor to the loss of seagrass beds (Bull *et al.* 2012; Delgado *et al.* 1997, 1999; Duarte 1995, 2002; Harlin, 1993; Hemminga and Duarte 2000; Orth *et al.* 2006; Short and Wyllie-Echeverria 1996; Touchette 2007; Van Katwijk *et al.* 1997; Vidal *et al.* 1999). Eutrophication of coastal waters as a result of nutrient runoff has been indicted as the most common contributor to declines in seagrass populations (Duarte 1995, 2002; Hemminga and Duarte 2000; Orth *et al.* 2006; Short and Wyllie-Echeverria 1996; Vidal *et al.* 1999). Seagrasses are adapted to thrive in low nutrient environments so the effects of eutrophication are generally indirect and have minimal benefits on the health of seagrasses (Borum and Sand-Jensen 1996). However, other primary producers

thrive on these supplemental nutrients and can outcompete healthy seagrasses (Duarte 1995). Phytoplankton can explode into massive algal blooms, thus limiting light availability and negatively impacting seagrass beds (Harlin, 1993; Touchette 2007). Excess nutrients can also stimulate epiphytic growth on seagrass leaf blades, essentially smothering them and resulting in death (den Hartog 1994; Duarte 1995, 2002; Hauxwell *et al.* 2001; Hemming and Duarte 2000). After the affected seagrasses have died, the roots and rhizomes, which are important in sediment stabilization, decompose, leading to sediment resuspension (Harlin 1993; Touchette 2007). These particulates in the water column reduce light availability and further weaken the remaining seagrasses in a devastating cycle of seagrass loss and sediment resuspension.

Fish farming and aquaculture are also examples of human activities which can have a direct negative impact on the health of seagrass beds and lead to subsequent declines (Orth *et al.* 2006). Seagrass meadows are often sought as prime locations for aquaculture due to their proliferation in shallow, protected areas (Delgado *et al.* 1997, 1999; Duarte 2002). However, some fish farming practices are detrimental to seagrass beds. Physical disturbance, nutrient overload, shading from fish enclosures and deposition of wastes all decrease light availability, essentially leading to suffocation of the seagrass bed (Delgado *et al.* 1997, 1999; Duarte 2002; Orth *et al.* 2006).

In addition to anthropogenic influences, although not mutually exclusive, biological and climatological factors have also been implicated as contributors to seagrass losses. Overgrazing by organisms such as sea urchins has been directly responsible for regional declines in seagrasses (Orth *et al.* 2006). The introduction of exotic species has also negatively affected seagrass communities. Of those exotic species

which have become established in seagrass beds, over half are documented as having negative impacts on seagrass ecosystems (Orth *et al.* 2006). Climate change has also had deleterious effects on the health of seagrass beds: increased sea levels have decreased light attenuation; increased sea surface temperatures have led to stressed seagrasses; and increased frequency and intensity of storms has led to mechanical damage of seagrasses due to surges and swells and an influx of fresh water (Orth *et al.* 2006).

The decline in seagrasses has not been a result of a single stressor, but rather, multiple stressors acting in concert both temporally and geographically (Orth *et al.* 2006). Like any organism, seagrasses are adapted to deal with changes in moderation. Throughout their evolutionary history, seagrasses have adapted to variations in water chemistry and temperature, shoreline morphology and sea-level fluctuations (Orth *et al.* 2006). Generally, these changes have been gradual, but the proliferation of changes in the past century has been rapid and the loss of seagrasses far exceeds recovery (Orth *et al.* 2006).

Massive die-off events of the recent past have had devastating consequences. A widespread loss of the temperate species *Zostera marina* (i.e. eelgrass) on the North Atlantic coast led to a collapse of scallop fisheries, a reduction in waterfowl populations and the extinction of the eelgrass limpet (Carlton *et al.* 1991; Orth *et al.* 2006; Rasmussen 1977). Some seagrass beds never fully recovered; those that did took nearly four decades to achieve previous population sizes (Blakesley *et al.* 2002; Short *et al.* 1987). Future losses are also predicted to negatively impact fish and shellfish species, including economically important ones (Robblee *et al.* 1991).

1.4 Wasting Disease

Disease is also a major contributor to seagrass loss both globally and temporally (Orth *et al.* 2006). Perhaps one of the better known, and well-studied, phenomenon related to seagrass declines is that of wasting disease (Figure 1). Wasting disease is characterized by brown patches of necrotic tissue on the seagrass blade (Bull *et al.* 2012; Burdick *et al.* 1993; den Hartog 1989) (Figure 2). What begin as small black or brown spots and streaks on the leaf blade quickly coalesce, spanning the width of the blade (Burdick *et al.* 1993; Cottam 1933a; Huntsman 1932; Muehlstein 1989; Ralph and Short 2002; Renn 1936a; Short *et al.* 1988; Van der Werff 1938; Young 1937). These large lesions decrease photosynthetic function by blocking transport of nutrients and photosynthates, eventually depleting the energy stores of the rhizomes ultimately leading to the death of the shoot (Muehlstein 1989; Renn 1936a; Young 1937). Because wasting disease is spread by blade to blade contact, infected blades that have been released from the rhizome or sheath are capable of further spreading the infection (Ralph and Short 2002).

The earliest accounts of seagrass decline were in the 1890's, but these incidents were not well documented (Cottam 1934a, 1935b; Muehlstein 1989). The first reliably recorded observations of seagrass declines in relation to wasting disease were in the 1930's among the temperate seagrass *Zostera marina* (i.e. eelgrass). Seagrass beds along the North American coastline were the first to exhibit mass mortality (Cottam 1933, 1935; Huntsman 1932; Renn 1934, 1935, 1936; Sullivan 2011). The epidemic, which possibly began in Virginia in 1930 (Huntsman 1932; Muehlstein 1989), had eliminated 90% of the eelgrass population along the Atlantic coast by 1931 (Cottam 1933a;

Muehlstein 1989; Tutin 1942). By 1932, loss estimates were around 99% (Cottam 1933a; Muehlstein 1989; Tutin 1942) and the epidemic had seemingly traversed the Atlantic with the first accounts of eelgrass populations succumbing to the disease in France (Sullivan 2011). From 1933-1935, the epidemic continued to spread on both sides of the Atlantic, reaching the Gulf of St. Lawrence in Canada (Cottam 1934a, b; Muehlstein 1989) and radiating throughout Europe among the United Kingdom, Sweden, Holland, Denmark and Germany (Cotton 1933; Petersen 1934; Sullivan 2011). By the late 1930's, eelgrass beds in British Columbia on the Pacific coast were beginning to decline (Muehlstein 1989) and in 1942 wasting disease was confirmed to have reached the California coast (Muehlstein 1989; Renn 1942).

In North America, the wasting disease outbreak peaked between 1930 and 1933 with the northeastern Atlantic coast populations suffering the greatest losses (Short *et al.* 1987). By 1938, die-offs were localized and some areas, such as Chesapeake Bay in Maryland, Shinnecock Bay in New York and parts of Virginia had almost fully recovered (Muehlstein 1989). However, in other areas, eelgrass was completely, and permanently, lost (Short *et al.* 1987). Despite brief reprises of wasting disease throughout the 1940's, populations capable of recovery were essentially reestablished by the 1950's (Cottam 1945; Cottam and Munro 1954; Muehlstein 1989; Short *et al.* 1987).

A second episode of wasting disease among *Z. marina* occurred in the 1980's with many similarities to the 1930's outbreak. The first reports of wasting disease came from Great Bay Estuary in New Hampshire followed in 1984 by almost complete loss of eelgrass in Cape Ann, Massachusetts (Short *et al.* 1986, 1987). The epidemic spread to the coasts of Maine, Connecticut, North Carolina, and reach Nova Scotia by 1987 (Short

et al. 1987). Similar to the first epidemic, the wasting disease was not restricted to the North American Atlantic coast, but also reached Europe and the Pacific. On the Pacific coast, eelgrass was lost in the Puget Sound and Washington State (Short *et al.* 1987; Burdick *et al.* 1993); in Europe, France reported die-offs associated with wasting disease (Short *et al.* 1987). During the 1980's outbreak, Moroiso Bay in Japan also suffered substantial loss (Burdick *et al.* 1993).

Both epidemics, in the 1930's and in the 1980's, had similar patterns of infection followed by mass mortality (Short *et al.* 1987). Eelgrass bed infection patterns were also comparable; seagrass beds that were the least affected were located in bays and estuaries with low salinity concentrations (Huntsman 1932; Muehlstein 1989; Short *et al.* 1987). During the first outbreak of the 1930's, the etiology of wasting disease was highly speculative and inconclusive (den Hartog 1987; Short *et al.* 1987). However, two theories garnered the most attention: 1) a microscopic organism was responsible for the outbreak, or 2) environmental stressors made the seagrasses more susceptible to disease by a secondary opportunistic pathogen (Petersen 1934; Rasmussen 1977; Renn 1935; Short *et al.* 1987). By the 1990's, researchers generally agreed that wasting disease is caused by a slime-mold like protist of the *Labyrinthula* genus, and that environmental stressors can render some populations of seagrasses more susceptible to infection than other populations (Blakesley *et al.* 2002; Peterson 1934; Renn 1935; Rasmussen 1977; Short *et al.* 1987).

Another episode of wasting disease also began in the 1980's in Florida Bay among the tropical seagrass *Thalassia testudinum* (i.e. turtle grass). Florida Bay is located at the southern end of Florida, bordered on the north by the Everglades National

Park and on the east by the Florida Keys (Figure 2). In the summer of 1987, the first observations of dead or dying turtle grass were made in Florida Bay and an estimated 4,000 hectares were lost while another 23,000 hectares were affected (Robblee *et al.* 1991). By the summer of 1988, approximately 30% of dense seagrass beds were gone (Robblee *et al.* 1991). The die-off continued through the early 1990's at an annual rate of 1 km^2 resulting in patchy beds (Carlson *et al.* 1994; Durako and Kuss 1994; Robblee *et al.* 1991; Thayer *et al.* 1994; Zieman *et al.* 1999). Akin to the die-off events relating to the temperate seagrass, *Z. marina*, the wasting episode in Florida Bay also varied in losses that seem to correlate with environmental factors. The worst die-off was in dense beds and shallow waters (Robblee *et al.* 1991). Additionally, die-off episodes were more rapid and severe in the Fall and Spring (Robblee *et al.* 1991).

The cause(s) of wasting disease were initially equivocal and debated. Early etiologies included poor environmental conditions due to both natural and anthropogenic sources. Since then, *Labyrinthula* spp., a slime mold like protist, has been isolated from affected seagrass shoots and identified through Koch's postulates as the causative agent of wasting disease (Bull *et al.* 2012; Burdick *et al.* 1993; Muehlstein *et al.* 1988, 1991; Porter and Muehlstein, 1989; Ralph and short 2002; Short *et al.* 1987; Vergeer *et al.* 1995).

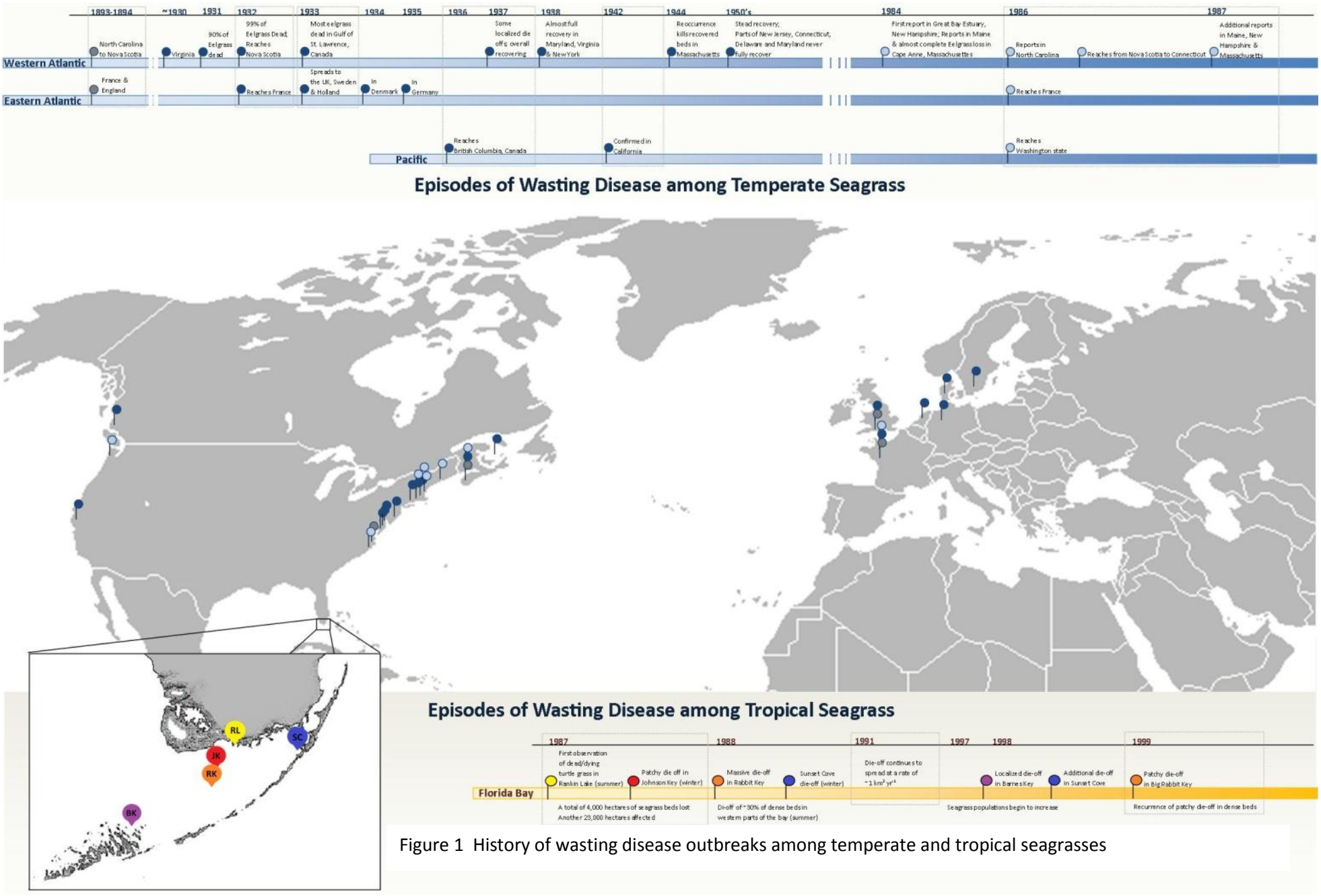

Figure 1 History of wasting disease outbreaks among temperate and tropical seagrasses

Figure 2 Necrotic lesion on *T. testudinum* caused by infection from *Labyrinthula* spp.

1.5 Labyrinthula spp.

Wasting disease in seagrasses is an example of a complex relationship between pathogens and marine species. *Labyrinthula* spp. (Figure 3), the protistan pathogens associated with seagrass wasting disease, are ubiquitous in marine environments around the globe (Muehlstein, 1992; Muehlstein *et al.* 1991; Short *et al.*, 1988; Vergeer *et al.* 1995; Verger and Develi 1997). There are multiple species in the *Labyrinthula* genus, many of which are host specific (Muehlstein *et al.* 1988, 1991; Ralph and Short 2002). Some species have been identified as primary pathogens (Blakesley *et al.* 2002; Mckone and Tanner 2009; Muehlstein *et al.* 1991; Ralph and Short 2002; Short *et al.* 1987; Steele *et al.* 2005), such as *L. zosterae*, which was responsible for the wasting disease epidemic of the 1980's among temperate eelgrass (Burdick *et al.* 1993; Muehlstein *et al.* 1988, 1991).

Although the mechanisms of *Labyrinthula* spp. infection are still unclear, basic elements of the infection process have been identified. *Labyrinthula* spp., and subsequently wasting disease, are spread by blade to blade contact and impair photosynthesis causing stress to the plant (Bull *et al.* 2012). After an enzymatic degradation of the cell wall, the Labyrinthulid enters the cell and destroys its cytoplasmic contents (Bull *et al.* 2012; Ralph and Short 2002). As the seagrass blade tissue dies, a necrotic lesion is formed and can eventually spread across the width of the blade. Even green, apparently "healthy" tissue surrounding the lesion site is physiologically stressed and has reduced photosynthetic capacity compared to non-infected shoots (Ralph and Short 2002). The dead tissue blocks vascular transport leading to further reduced

photosynthetic efficiency (Durako and Kuss 1994; Ralph and Short 2002; Renn 1936) and eventually death.

Despite past wasting disease events, which resulted in massive seagrass die-offs, virulent *Labyrinthula* spp. have been isolated from seagrass beds that did not succumb to such fate (Short *et al.* 1988; Vergeer *et al.* 1995; Vergeer and Den Hartog 1994). It is still unknown what factors actually contribute to large-scale outbreaks that result in wide spread die-off (Ralph and Short 2002). Some have suggested the presence of *Labyrinthula* spp. alone is not enough to cause mass mortality in healthy seagrass beds (Ralph and Short 2002; Rasmussen 1977; Tutin 1938; Verger and den Hartog 1994; Young 1937); however, it has been purported that healthy plants can resist infection while stressed ones are more vulnerable and easily succumb to wasting disease (Den Hartog 1996; Renn 1937; Tutin 1938; Vergeer *et al.* 1995; Verger and Develi 1997). Seagrass infected with *Labyrinthula* spp. not only exhibit reduced photosynthetic efficiency, but also negative carbon balance and other physiological stressors (Durako and Kuss 1994). Any additional stress, such as adverse environmental conditions, may severely impact the survival of the seagrass and result in death (Ralph and Short 2002). Given the right conditions, such as dense seagrass beds with long-term exposure to environmental stressors, and wasting disease may result in a wide spread epidemic (Robblee *et al.* 1991).

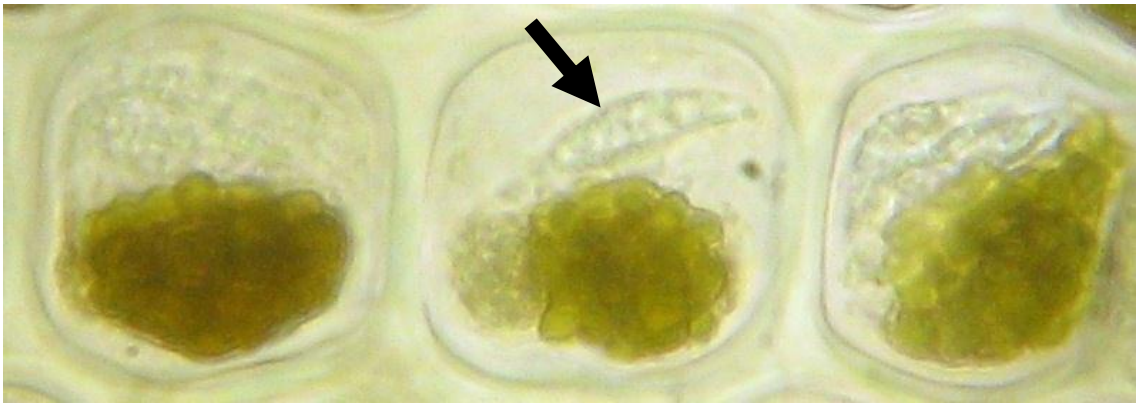

Figure 3 In situ localization of spindle-shaped *Labyrinthula* sp. cells inside epidermal cells of the seagrass *T. testudinum*

1.6 Environmental Stressors

Environmental stressors are among the most commonly suggested antagonists of chronic stress in seagrass beds; because seagrass distribution is limited by light, salinity, temperature and nutrients (Mazzotti *et al.* 2007), a severe and prolonged shift in any of these components could lead to stressed beds. Indeed, environmental stressors, such as salinity, temperature, sulfide, and hypoxic conditions have all been correlated with die-off events (Koch *et al.* 2007a; Robblee *et al.* 1991).

Salinity

Salinity is an important abiotic factor affecting the health of seagrass communities (Montague and Ley 1993; Touchette 2007). Although most seagrasses can grow in estuarine or brackish waters, many need higher salinities to propagate (Duarte 2002; Hemminga and Duarte 2000). The optimum salinity range for *Thalassia testudinum*, the climax species of seagrass in Florida Bay, is between 24-35 ppt (Mazzotti *et al.* 2007; Zieman and Zieman 1989) with maximum photosynthetic efficiency positively correlated with higher salinity (Mazzotti *et al.* 2007). At lower salinities (~17ppt), *T. testudinum's* photosynthetic efficiency is reduced and growth, evidenced by blade length and production, also ceases (Dawes *et al.* 2004; Mazzotti *et al.* 2007).

Although seagrasses are adapted to cope with dynamic saline environments, drastic fluctuations in salinity can adversely affect seagrass health (Dawes *et al.* 2004; Tomasko and Hall 1999). Salinity stress is mainly due to the influx of toxic Na^+ ions. In high concentrations, these ions can lower cell membrane potentials, disrupt metabolism, lead to a loss of osmotic balance and decrease photosynthetic efficiency (Touchette 2007;

Trevathan *et al.* 2011). Specifically, elevated salinities can inhibit photosynthesis by interrupting the electron transport chain, leading to the production of reactive oxygen species (ROS) (Touchette 2007). If these toxic ROS are not amended by the plant, they can cause further damage to the photosystems by oxidizing important membranes, photosynthetic pigments and enzymes (Touchette 2007).

In Florida Bay, salinity stress has been a proposed component of massive die-off events associated with wasting disease. In some areas, high temperatures (leading to evaporation) and seasonal droughts resulted in salinities ranging from 50-70 ppt (Boyer *et al.* 1999; Kelble *et al.* 2007; Koch *et al.* 2007b; Robblee *et al.* 1991; Trevanthan *et al.* 2011). Laboratory based studies have shown that hypersaline conditions such as these elicit a stress response in *T. testudinum* and that prolonged exposure results in mortality (Kahn and Durako 2006). As such, chronic hypersalinity may have contributed to die-off events by stressing the seagrasses, thus making them more susceptible to infection by *Labyrinthula* spp. (Trevathan *et al.* 2011). In Florida Bay, areas of high salinity displayed greater prevalence of wasting disease (Blakesley *et al.* 2002; Trevathan *et al.* 2011).

Although hypersaline conditions have been associated with wasting disease events, seagrasses are not the only organism affected by fluctuating salinities. *Labyrinthula* spp., the causal organisms of wasting disease, are also sensitive to extreme saline conditions. In Florida Bay, no wasting was observed in areas with salinities below 15ppt (Blakesley *et al.* 2002; Trevathan *et al.* 2011). This correlation was corroborated by laboratory studies which revealed that salinity directly impacts the growth and virulence of *Labyrinthula* spp. (Martin *et al.* 2009; McKone and Tanner 2009;

Muehlstein *et al.* 1988; Trevathan 2011; Young 1943). It is hypothesized that extreme salinities (<15ppt & >50ppt) may affect the ectoplasmic network of *Labyrinthulal* spp., therefore making it difficult to infect and/or adhere to the host seagrass (Trevathan *et al.* 2011).

Temperature

Studies on thermal tolerance and adaptations for marine plants are limited in comparison to terrestrial studies (Bergmann *et al.* 2010), However, temperature is one of the most influential and rapidly changing abiotic factors in seagrass communities (Dawes *et al.* 2004; Short and Neckles 1999). For tropical seagrass species (e.g. *Thalassia testudinum*) the optimal temperature is approximately 30°C with an upper threshold ranging between 33-36°C and a lower threshold at approximately 15°C (Chamberlain 2004; Koch and Erskine 2001; Koch *et al.* 2007a; Mazzotti *et al.* 2007; Zieman 1971; Zimmerman and Livingston 1976). At temperatures outside of their optimal range, productivity decreases and seagrasses exhibit defoliation and reduced growth (Bull *et al.* 2012; Durako and Moffler 1985; Mazzotti *et al.* 2007; McMillan 1978; Moore and Short 2007; Zimmerman and Livingston 1976).

Temperature has been hypothesized to affect a multitude of processes including photosynthesis and respiration, growth and reproduction, and nutrient uptake (Duarte 2002; Short and Neckles 1999). For example, water temperature, not photoperiod, is primarily responsible for flowering in *T. testudinum* (Dawes *et al.* 2004; McMillan 1982; Moffler and Durako 1987). At higher temperatures, photosynthetic capacity and

efficiency is reduced as the rate of biochemical reactions is altered and photosynthesis wanes (Bulthuis 1987; Campbell *et al.* 2006; Koch *et al.* 2007a; Mazzotti *et al.* 2007).

Although infection by *Labyrinthula* spp. is the primary cause of wasting disease, what remains to be determined is why some outbreaks are more drastic than others (Bull *et al.* 2012; Burdick *et al.* 1993). When considering the effects of thermal stress, many have hypothesized that elevated temperatures have led to an increase in wasting disease episodes and intensity because stressed seagrasses are unable to cope with the added pressure of infection from *Labyrinthula* spp. (Blakesley *et al.* 2002; Carlson *et al.* 1994; Dawes *et al.* 2004; Durako 1994; Durako and Kuss, 1994; Durako *et al.* 2001; Robblee *et al.* 1991). Some have proposed that temperature has actually regulated the scale at which outbreaks have occurred, with seagrasses in optimal temperature ranges experiencing only localized and patchy die-off, while extreme temperatures may have resulted in large scale epidemics (Bull *et al.* 2012; den Hartog 1989; Rasmussen 1977).

Indeed, temperature has often been indicted as a trigger for disease outbreak (Bull *et al.* 2012; Rasmussen 1970). In Florida Bay, temperatures exceeding the optimal range for *T. testudinum* have been recorded and associated with beds that experienced some of the most extreme die off (Blakesley *et al.* 2002). The virulence of wasting disease is also correlated with seasonal variations (Blakesley *et al.* 2002). In the spring and early summer months, infection from *Labyrinthula* spp. is minimal. However, as the year progresses and the water temperature increases, the prevalence of wasting disease also increases, resulting in peak infection rates in the late fall (Blakesley *et al.* 2002). Global ocean temperatures have increased by an average of 0.74^{o}C over the past 100 years and are expected to increase 1-4^{o}C by the end of the next century (Hoegh-Guldberg *et al.*

2007; IPCC 2007; Przeslawski *et al.* 2008). Many seagrass species are already at the upper limits of their physiological range (Campbell *et al.* 2006; Ralph 1998). Thus, slight increases in temperature may serve as a tipping point pushing seagrasses past their thermal thresholds and enhance susceptibility to disease.

Sulfide

Sulfides have also been hypothesized to contribute to seagrass die-off. Hydrogen sulfide (H_2S), a prevalent species of porewater sulfides, is toxic to many plants and animals, including seagrasses (Bagarinao 1992; Bradley and Dunn 1989; Carlson *et al.* 1994; Goodman *et al.* 1995; Havill *et al.* 1985; Holmer and Bondagaard 2001; Joshi *et al.* 1975; Koch and Mendelssohn 1989; Koch *et al.* 1990, 2007a, b). Sulfides are general cell poisons that inactivate important metabolic enzymes and inhibit the uptake and assimilation of nutrients and oxygen, eventually leading to death (Vamos and Koves 1972).

The formation of sulfides is the result of sulfate reducing anaerobic bacteria in the sediment (Dawes *et al.* 2004). As organic materials are oxidized by microorganisms, sulfate is reduced to sulfide (Koch *et al.* 2007a). If the toxic sulfides are not oxidized back to sulfates or bound into a solid form (e.g. pyrite FeS_2), the concentration of porewater sulfides can reach lethal levels (Koch *et al.* 2007a). Because many of the carbonate sediments in which tropical seagrasses grow are low iron, the formation of pyrite is rare and plants must oxidize their rhizosphere to prevent sulfide intrusion of their tissues (Koch *et al.* 2007a). Therefore, a plant's tolerance of sulfide is based upon its ability to oxidize sulfide back to sulfate (Goto and Tai 1957; Vamos and Koves 1972).

Numerous biotic and abiotic factors determine the rate at which sulfides will accumulate in the sediment. These include temperature, microbial respiration rates, sediment composition and oxidizing capacity of associated organisms (Barber and Carlson 1993; Berner 1984; Carlson *et al.* 1994; Eldridge *et al.* 2004; Hines and Lyons 1982; Holmer *et al.* 2003; Koch *et al.* 2007a; Oremland and Taylor 1976). Oxidation and reduction of sulfur is regulated by temperature (Koch *et al.* 2007a; Wieland and Kuhl 2000). High temperatures generally lead to higher rates of sulfate reduction because bacteria respiration rates are mediated by temperature (Barber and Carlson 1993; Carlson *et al.* 1994; Koch *et al.* 2007a, b). However, anaerobic bacteria are limited by the availability of organic compounds. Sediments in seagrass ecosystems are generally high in labile carbon due to the seagrass' extensive root and rhizome network which traps organic matter (Hemminga and Duarte 2000; Koch *et al.* 2007a; Papenbrock 2012), thus contributing to higher sulfide concentrations.

Fortunately, seagrasses have evolved many adaptations that prevent the accumulation of toxic sulfides. Seagrasses, such as *T. testudinum*, are highly efficient at oxidizing their rhizosphere due to an extensive network of gas conducting tissue that extends between their leaf blades and rhizome (Armstrong 1979; Borum *et al.* 2005; Carlson *et al.* 1994; Koch *et al.* 2007a). The aerenchyma transport oxygen produced from photosynthesis to below ground tissues and oxidize sulfide back to sulfate, effectively protecting their rhizosphere from sulfide intrusion (Eldridge *et al.* 2004; Koch *et al.* 2007a; Tomlinson 1969). *Thalassia testudinum* has been shown to not only tolerate, but continue growth, with porewater sulfide concentrations up to 10mM (Erskine and Koch 2000; Koch *et al.* 2007a, b). In contrast, short term exposure to 0.3mM sulfide

was lethal for *Halophila engelmanni*, an early successional species of seagrass beds (Erskine and Koch 2000).

Despite their ability to inhabit highly reduced sediments, some have suggested that high sulfide concentrations, especially in concert with other abiotic stressors, may play a role in seagrass die-off in relation to wasting disease (Carlson *et al.* 1994; Koch *et al.* 2007b). It is hypothesized that high sulfide levels contributed to die-off events in Florida Bay where the sulfide levels of infected beds ranged from 5.7mM to over 13mM (Carlson *et al.* 1994; Koch *et al.* 2007a; Robblee *et al.* 1991). In comparison, healthy beds had sulfide concentrations lower than 2mM (Carlson *et al.* 2002; Dawes *et al.* 2004). Empirical studies have shown that diseased and/or stressed plants actually release organic exudates, which further promotes sulfate reduction resulting in increased porewater sulfide concentrations (Hines *et al.* 1999; Koch *et al.* 2007a). In these scenarios, stressed beds may have further contributed to die-off events in a cyclic fashion.

Sulfide is often considered a secondary stressor (Carlson *et al.* 1994). In laboratory studies, mortality has only been observed when combined with other stressors, such as high salinity and high temperature (Koch and Erskine 2001). In relation to wasting disease, the combination of sulfide and other stressors may contribute to die-off by making the seagrasses more susceptible to infection by *Labyrinthula* spp. (Carlson *et al.* 1994).

The Role of Multiple Stressors

The number of studies examining the role of multiple stressors, especially in relation to wasting disease, is limited (Orth *et al.* 2006). High temperatures, high salinity

and high porewater sulfide concentrations have all contributed to die-off events (Borum *et al.* 2005; Carlson *et al.*, 1994; Koch and Erskine 2001; Koch *et al.* 2007a, b, c; Robblee *et al.* 1991; Zieman *et al.* 1999), however, these abiotic stressors are not mutually exclusive and often occur in concert with one another. For example, high temperatures may increase salinity due to evaporation and/or increase sulfide concentrations by speeding up microbial reduction rates. Therefore, it is imperative to examine the interacting effects of multiple stressors.

Some laboratory based studies have tested *T. testudinum's* response to different combinations of abiotic stressors. Although *T. testudinum* is fairly tolerant to thermal stress, a combination of high temperatures and sulfide may disrupt carbon metabolism (Koch *et al.* 2007a). Thalassia testudinum also appears to be more tolerant than other tropical species of seagrasses to the combination of high temperatures and hypoxia (Koch *et al.* 2007a). However, when sulfide and salinity stress are induced, there appears to be a negative synergistic effect on the oxygen balance of *T. testudinum* (Koch *et al.* 2007b). In a study by Koch and Erskine (2001) the effects of elevated temperatures, hypersalinity and sulfide were examined. By itself, sulfide did not affect *T. testudinum's* growth or leaf O_2 production, even in concentrations as high as 10mM. However, when sulfide (6mM) was combined with either salinity (56ppt) or thermal stress (35°C), die-back of seagrasses was observed. When all three stressors were combined, the mortality rate was at 100%, indicating that multiple stressors may act synergistically.

1.7 Objectives and Hypotheses

The purpose of this study was to explicitly test how the combined effects of multiple stressors impact *T. testudinum* health and susceptibility towards *Labyrinthula* spp. infection under controlled conditions. A series of experiments were conducted, each testing a different combination of ecologically relevant abiotic stressors that are known to impact seagrass health. Chapter 2 examines various combinations of stressors under both a recovery simulation and one in which the stressors were maintained throughout. The effects of salinity, temperature, sulfide and hypoxia were each examined in a full-factorial design. The growth of *Labyrinthula* sp. was also examined under the combination of salinity and temperature stress.

Hypothesis 1: Seagrasses incubated under the application of a given stressor will show evidence of declining health and, in turn, will be more prone to infection, as quantified by lesion size.

Hypothesis 2: The application of combined stressors will result in an additive or multiplicative effect with respect to pathogen susceptibility and lesion size. It is anticipated that treatment groups with multiple stressors will not only exhibit larger lesions, but that the effects of these multiple stressors will be synergistic, not simply additive.

These results are especially important in a time where monitoring and managing environmental change are prevalent among the scientific community in that they will allow a greater understanding of how combined environmental stressors affect *T. testudinum's* susceptibility to infection from *Labyrinthula spp*.

2

Effects of multiple abiotic stressors on the health and susceptibility of *Thalassia testudinum* to wasting disease when exposed to *Labyrinthula* spp.

2.1 Introduction

Seagrass beds are highly productive ecosystems that substantially contribute to the economic and ecological welfare of many marine habitats (Dawes *et al.* 2004; Duarte 2002; Orth *et al.* 2006; Papenbrock 2012). In Florida alone, seagrass beds support a multi-million dollar recreational and commercial fishing and boating industry by providing essential habitat to a biodiverse faunal assemblage (Dawes *et al.* 2004; Hemminga and Duarte 2000; Mazzotti *et al.* 2007; Orth *et al.* 2006; Robblee *et al.* 1991). Indeed, many commercially important fish species relies on seagrass habitat at some point in their life (Dawes *et al.* 2004). Aside from the multitude of roles associated with organismal use, seagrass beds are important ecological engineers, mitigating their surrounding environment physically and chemically (Orth *et al.* 2006; Papenbrock 2012). Both above and below ground tissues play important roles in water quality and sediment stabilization, primary production and nutrient cycling (Dawes *et al.* 2004; Duarte 2002; Hemminga and Duarte 2000; Mazzotti *et al.* 2007; Orth *et al.* 2006).

Unfortunately, seagrasses are experiencing declines worldwide (Bergmann *et al.* 2010; Bull *et al.* 2012; Duarte 2002; Orth *et al.* 2006; Papenbrock 2012; Touchette 2007). Multiple interacting stressors, global and local, biotic and abiotic, human and natural, are causing declines in seagrass populations (Orth *et al.* 2006). The loss of seagrasses far exceeds the rate of increase or recovery (Orth *et al.* 2006). Previous losses of seagrass

habitat have had drastic effects and are associated with the collapse of scallop fisheries, reductions in waterfowl populations and extinction (Carlton *et al.* 1991; Orth *et al.* 2006; Rasmussen 1977).

One of the leading causes of seagrass declines is a phenomenon known as "wasting disease". Wasting disease is characterized by brown/black necrotic lesions on the seagrass blade. As the seagrass cells die, the lesion increases in size, effectually reducing photosynthetic efficiency and eventually leading to death (Muehlstein 1989). Major outbreaks of the disease have occurred in the past century with recovery spanning over four decades (Blakesley *et al.* 2002; Short *et al.* 1987). The first recorded outbreak was in the 1930's and occurred among the temperate seagrass species, *Zostera marina*. The devastation was global in scale and some seagrass beds never fully recovered. Another episode in the 1990's was among the tropical seagrass species, *Thalassia testudinum*, and was prevalent in the Florida Bay region. Approximately 4,000 hectares of seagrasses were lost and another 23,000 hectares were negatively impacted, presumably succumbing to wasting disease (Robblee *et al.* 1991).

The causal agent of wasting disease is a slime-mold like protist in the family Labyrinthulaceae (Bull *et al.* 2012; Muehlstein *et al.* 1991; Ralph and Short 2002; Short *et al.* 1987). *Labyrinthula* spp. are ubiquitous in the marine environment and each species of seagrass has its own species of Labyrinthulid associated with it (Verger and Develi 1996; Verger and den Hartog 1994). Some species have been identified as primary pathogens (Blakesley *et al.* 2002; Mckone and Tanner 2009; Muehlstein *et al.* 1991; Ralph and Short 2002; Short *et al.* 1987; Steele *et al.* 2005), such as *L. zosterae*,

which was responsible for a wasting disease epidemic in the 1980's among temperate eelgrass (Burdick *et al.* 1993; Muehlstein *et al.* 1988, 1991).

Despite past wasting disease events, which resulted in massive seagrass die-offs, virulent *Labyrinthula* spp. have been isolated from seagrass beds that did not succumb to such fate (Short *et al.* 1988; Vergeer *et al.* 1995; Vergeer and Den Hartog 1994). For this reason, some species are hypothesized to act as secondary opportunistic pathogens (Short *et al.* 1987). However, it still unclear as to what factors actually contribute to large-scale outbreaks of wasting disease that result in wide spread seagrass die-off.

Some have suggested that environmental stressors, such as hypersalinity, increased temperatures, high concentrations of sediment sulfide and hypoxia may have contributed to recent die-off events by decreasing *Thalassia testudinum's* ability to resist infection from *Labyrinthula* spp. (Ralph and Short 2002; Ramsussen 1977; Tutin 1938; Young 1937). In Florida Bay, areas of high salinity displayed greater prevalence of wasting disease (Blakesley *et al.* 2002; Trevathan *et al.* 2011). Temperature has also been indicted as a trigger for disease outbreak (Bull *et al.* 2012; Rasmussen 1970). In Florida Bay, temperatures exceeding the optimal range for *T. testudinum* have been recorded and associated with beds that experienced some of the most extreme die off (Blakesley *et al.* 2002). Sulfide may also contribute to die off by acting as a secondary stressor. Infected seagrass beds in Florida Bay had sulfide concentrations well over 13mM in comparison to healthy beds whose sulfide concentrations did not exceed 2mM (Carlson *et al.* 1994; Koch *et al.* 2007a; Robblee *et al.* 1991). Although *T. testudinum* has demonstrated a high tolerance to sulfide toxicity due to its ability to utilize photosynthetically produced oxygen to oxidize its rhizosphere, the combination of sulfide

and other stressors may contribute to wasting disease episodes. For example, it has been demonstrated that hypoxic water conditions reduce seagrasses' ability to oxidize their rhizospheres, and may ultimately result in sulfide poisoning (Koch et al 2007b). Additionally, laboratory studies have demonstrated that seagrasses exposed to sulfide, hypersalinity and elevated temperatures experienced high mortality rates, even without the presence of *Labyrinthula* spp. (Koch *et al.* 2007b). Despite these observations, there is a lack of research examining the effects of combined stressors in relation to wasting disease.

Limited studies have tested *Labyrinthula* spp.'s response to environmental stressors. Martin *et al.* (2009) demonstrated that *Labyrinthula* sp. colony size is reduced at both hypo- (<10 ppt) and hyper- (>50 ppt) salinities. In surveys of Florida Bay, areas with salinities below 15 ppt were unaffected by wasting disease (Blakesley *et al.* 2002).

The purpose of this study was to explicitly test how the combined effects of elevated salinity, elevated temperature, sulfide and night-time hypoxia impact *T. testudinum* health and susceptibility towards *Labyrinthula* spp. infection under controlled conditions. It was hypothesized that seagrasses incubated under a given stressor would show evidence of declining health and, in turn, would be more prone to infection, as quantified by lesion size. As such, the application of multiple stressors would result in an additive or multiplicative effect with respect to pathogen susceptibility and lesion size. It was anticipated that treatment groups with elevated salinity and elevated temperature would not only exhibit larger lesions and decreased photosynthetic efficiency, but that the effects of these multiple stressors would be synergistic, not simply additive.

2.2 Materials and Methods

Collection and Maintenance of T. testudinum and Labyrinthula sp.

T. testudinum was collected off the Gulf coast of Florida (29°20'N, 83°23'W), cleaned of epiphytes and subsequently transplanted into terra cotta pots filled with Arag-Alive!™ (CaribSea Inc., Ft. Pierce, FL). Plants were maintained in aquaria under greenhouse conditions at the University of North Florida, Jacksonville, FL at a salinity of 30 ppt, representative of the initial collection site. Aquaria diel temperature values were maintained between 25 and 27 °C and photosynthetic active radiation levels were < 300 μmol $m^{-2}s^{-1}$. Partial water changes were conducted on a weekly basis to maintain adequate nutrients. Specimens were allowed to acclimate for no less than one week prior to use in any experiment.

A known virulent strain of *Labyrinthula* sp. (Trevathan *et al.* 2011) was maintained in culture and used for all experiments described herein. Serum-seawater agar (SSA) described in Trevathan *et al.* (2011) was used for *Labyrinthula* sp. culture and contained 500 mL of prepared seawater (25 ppt; Instant Ocean® Sea Salt) combined with 6 g agar, 0.5 g glucose, 0.05 g nutritional yeast, 0.05 g peptone, 1.5 mg germanium dioxide, 12.5 mL streptomycin/penicillin (stock: 1.25 g streptomycin + 1.25 g penicillin per 100 mL de-ionized H_2O), and 5 mL horse serum. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

General Experimental Design

To evaluate the effects of independent and combined stress on the susceptibility of *T. testudinum* to *Labyrinthula* sp. infection several series of experiments were conducted.

Two experiments tested *T. testudinum's* response to infection under varying salinity and temperature; one experiment tested *T. testudinum's* response to infection under hypoxic conditions and sulfide stress; and two experiments tested *T. testudinum's* response to infection under a combined regime of hypoxic conditions, salinity, temperature, and sulfide stressors (Table 1). For experiments that were conducted as sets, one experiment maintained stress conditions throughout the duration of the experiment, while the other experiment simulated a recovery scenario in which the seagrasses were stressed and then returned to ambient conditions prior to infection with *Labyrinthula* sp.

Experimental microcosms (3.8L total volume) consisted of polyethylene terephthalate containers (Rubbermaid, Winchester, VA, USA). Full spectrum lighting was utilized using Power-FLO T5 HO bulbs (Hagen, West Yorkshire, UK) and was maintained at a 12:12 h light:dark photoperiod.

In all experiments seagrasses were randomly assigned a treatment group and incubated under their respective stressor(s) for seven days at which time their photosynthetic efficiency was measured (discussed below). With each set of stressors, seagrasses were either returned to their respective treatment group or returned to ambient conditions. They were then infected with *Labyrinthula* sp. according to methods established by Steele *et al.* (2005). Briefly, sterile segments (2cm) of *T. testudinum* were incubated for one week on prepared plates of *Labyrinthula* sp. Vectors were attached to

the second rank leaf of each replicate using a clamp made from segments of Tygon tubing. The control group received sterile vectors (i.e. without *Labyrinthula* sp.). Plants were then left to incubate for several days until visible signs of infection were evident (i.e. necrotic lesions). At that time, photosynthetic efficiency and lesion size and were quantified (discussed below). During the experiment, all microcosms were monitored so that stressors remained within the experimental parameters. De-ionized water was added as needed to account for evaporation. Microcosms were also individually aerated throughout the experiment to prevent hypoxic conditions where applicable.

Elevated Salinity and Elevated Temperature

To examine the effect(s) of elevated salinity (45 ppt) and elevated temperature (30°C) on the ability of *T. testudinum* to resist infection from *Labyrinthula* sp., shoots were randomly assigned to one of four treatment groups plus a control group exposed to a sterile vector (Table 1). Each treatment contained five replicates for a total of 25 microcosms. Salinity and temperature of each microcosm were monitored daily.

Prepared seawater was amended with Instant Ocean® Sea Salt to achieve elevated salinities when applicable. Elevated temperatures were achieved with individual 25watt Aqueon™ aquarium heaters that were added to the appropriate microcosms.

Night-time Hypoxia and Sulfide

To examine the effect(s) of night-time hypoxia (<2.00 mg/L) and sulfide (6 mM) on the ability of *T. testudinum* to resist infection from *Labyrinthula* sp., shoots were randomly assigned to one of four treatment groups, plus a control group exposed to a sterile vector (Table 1). Each treatment contained five replicates for a total of 25

microcosms. Ambient salinity and temperature were maintained throughout the experiment.

To restrict sulfide exposure to below ground tissue, root chambers (Figure 4) were constructed as per Koch and Erskine (2001) with minor modification. Briefly, seagrass short shoots were threaded through 4-5mm holes in rubber stoppers and were sealed on non-photosynthetic tissue using marine epoxy (West Marine®). Stoppers with shoots were then inserted into glass vials (300 mL) which served as root chambers and contained the appropriate sulfide concentration. Root chambers with shoots were then placed in individual microcosms. During the light cycle of the photoperiod, all microcosms were aerated. At the beginning of the dark cycle, N_2 gas was bubbled into the appropriate microcosms until hypoxic conditions were obtained. Normoxic treatments were continuously aerated.

Sulfide speciation is pH dependent. At the ambient pH of seawater (~8.2) the dominant sulfide species is the hydrosulfide anion (HS^-). Although HS^- is toxic at high concentrations (Koch and Erskine 2001), hydrogen sulfide is a potent phytotoxin and is the dominant sulfide species in seagrass sediments in Florida. At a pH of 7.0, the ratio of HS- to hydrogen sulfide (H_2S) is 50:50. Therefore, sodium sulfide ($NaS•9H_2O$) was dissolved in deoxygenated seawater that had an adjusted pH of 7.0 to obtain a hydrogen sulfide concentration of 6 mM. Sulfide concentrations in the root chambers were monitored with a solid-state silver/sulfide probe (Model 27504-28, Cole-Palmer®). Additionally, the seawater of the microcosm was monitored to ensure sulfide was restricted to root chambers. To account for sulfide oxidation, root chambers received fresh seawater/sulfide mixture every three days and were maintained at a sulfide

concentration of 6mM. Shoots in treatments that were not exposed to sulfide were still sealed in root chambers whose below ground tissues were exposed to deoxygenated seawater with a pH of 7.0. These chambers also received fresh seawater every three days to prevent nutrient limitation.

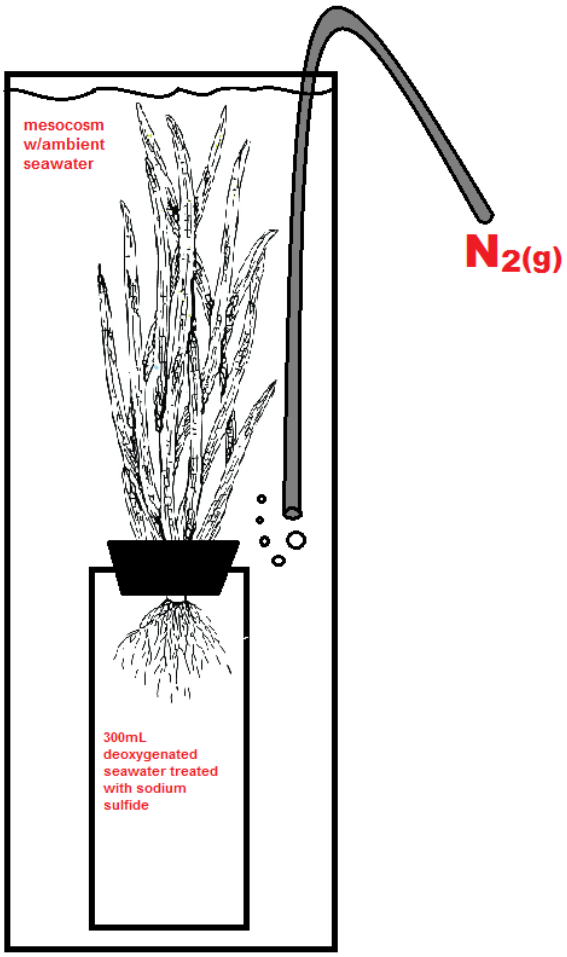

Figure 4 Experimental microcosm with root chamber containing seawater treated with sodium sulfide

Elevated Salinity, Elevated Temperature, Night-time Hypoxia and Sulfide

To evaluate the effect(s) of elevated salinity and temperature in combination with night-time hypoxia and sulfide on the susceptibility of *T. testudinum* to infection from *Labyrinthula* sp., a full-factorial design was utilized. Shoots were randomly assigned to one of sixteen treatment groups, plus a control group exposed to a sterile vector (Table 1). Each treatment contained five replicates for a total of 85 microcosms. Individual microcosms were utilized and experimental conditions were obtained using previously described methods.

Table 1 Experimental conditions

Experiment	Parameters[†]	Treatment Groups
1a (sustained stress) 1b (recovery simulation)	salinity + temperature	ambient salinity/ambient temperature ambient salinity/high temperature high salinity/ambient temperature high salinity/high temperature high salinity/high temperature (control)
2a	sulfide + hypoxia	no sulfide/normoxic no sulfide/hypoxic sulfide/normoxic sulfide/hypoxic sulfide/hypoxic (control)
3a (sustained stress) 3b (recovery simulation)	salinity + temperature + sulfide + hypoxia	ambient salinity/ambient temperature/no sulfide/hypoxia ambient salinity/ambient temperature/no sulfide/normoxic ambient salinity/high temperature/no sulfide/hypoxia ambient salinity/high temperature/no sulfide/normoxic ambient salinity/ambient temperature/sulfide/hypoxic ambient salinity/ambient temperature/sulfide/normoxic ambient salinity/high temperature/sulfide/hypoxic ambient salinity/high temperature/sulfide/normoxic high salinity/ambient temperature/no sulfide/hypoxic high salinity/ambient temperature/no sulfide/normoxic high salinity/high temperature/no sulfide/hypoxic high salinity/high temperature/no sulfide/normoxic high salinity/ambient temperature/sulfide/hypoxic high salinity/ambient temperature/sulfide/normoxic high salinity/high temperature/sulfide/hypoxic high salinity/high temperature/sulfide/normoxic high salinity/high temperature/sulfide/hypoxic (control)

[†] *ambient salinity (30 ppt) high salinity (45 ppt); ambient temperature (25 °C) high temperature (30 °C); no sulfide (0 mM) sulfide(6 mM); normoxic (> 8.00 mg/L) hypoxic (< 2.00 mg/L)*

Pulse Amplitude Modulated Fluorometry

To determine if the application of a given abiotic stressor or *Labyrinthula* sp. exposure induced a reduction in plant health, photochemical efficiency of each plant was measured using pulse amplitude modulated (PAM) fluorometry (Heinz-Walz GmbH ©, Effeltrich, Germany). The PAM fluorometer measures the efficiency of photosystem II by fully reducing the electron transfer chain with one saturating pulse of light. Because the receptors are full (i.e. fully reduced), energy cannot be transferred to the electron transfer chain and must either be released as heat (which is negligible) or fluoresce back at a lower wavelength. Measurements of fluorescence yield before and after the saturating pulse are taken and effective quantum yield (EQY) is measured using the following:

$$\text{Yield} = \frac{F_m' - F}{F_m'} = Fv/F_m$$

where F = fluorescence before the saturating pulse, and, F_m' = fluorescence directly after the saturating pulse.

Effective quantum yield values were utilized with the following parameters: measuring intensity = 5, gain = 6, damp = 2, saturation intensity = 2. To obtain measurements, a dark leaf clip (DIVING-LC) was attached 1 cm above the site of infection for consistency in holding the fiber optic cable 4 mm above the leaf surface for every measurement (Durako and Kunzelman 2002). Effective quantum yield measurements were taken prior to infection and at the close of the experiment to assess effects of infection on photosynthetic efficiency.

Lesion Measurements

Post treatment measurements of necrotic lesions were utilized as a proxy indicator of plant susceptibility to infection. Photographs of each shoot were taken using a Canon Powershot SX260 HS digital camera and lesion size for each plant was measured and quantified using ImageJ software (Rasband 1997-2012). Lesion measurements were reported in square centimeters (cm^2).

In Vitro Labyrinthula sp. Growth Assay

To assess the effects of salinity and temperature on culture growth of *Labyrinthula* sp., four treatment groups were prepared that mimicked the environmental conditions of the *T. testudinum* experiments: 1) ambient salinity (30 ppt)/ambient temperature (25°C); 2) ambient salinity/high temperature (30°C); 3) high salinity (45 ppt)/ambient temperature; 4) high salinity/high temperature. A 6 mm diameter cork borer was used to extract standard sized SSA plugs of *Labyrinthula* sp. from the growing edge of cultures incubated under conditions aforementioned in the collection and maintenance section. Each plug was placed surface side down into a 12 well microplate (Costar ®, Corning Inc., Corning, NY, USA) and randomly assigned to one of the treatment groups. Two mL of liquid media was then carefully added to each well. The liquid media was prepared according to the SSA recipe, but was augmented with salinities corresponding to treatment groups and the agar component was omitted. To obtain elevated temperatures, the microplates were immersed in a water bath.

After a 72 hour incubation period, the microplates were removed from their treatment groups and the liquid media and agar plug were carefully discarded.

Labyrinthula sp. cells were stained using 1 mL of 0.1% Crystal Violet histological stain (Fisher Scientific, Fair Lawn, NJ, USA). After one minute, the stain was removed, rinsed with de-ionized water and dried at 37°C for 30 minutes. The microplates were then inverted and the colony edge was traced for each well. Photographs of each well were taken using a Canon Powershot SX260 HS digital camera and colony size was measured and quantified in square millimeters (mm^2) using ImageJ software (Rasband 1997-2012).

Statistical Analyses

All statistical analyses were performed with 95% confidence intervals. Unless otherwise noted, data were normal and assessed with a Shapiro-Wilk test. A Levene's test was utilized to assess the equality of error variances among groups.

Elevated Salinity + Elevated Temperature (Stressors Maintained and Recovery Simulation)

For both experiments (stressors maintained and recovery simulation) a two-way ANOVA was conducted to test the effect of salinity and temperature on lesion size associated with infection from *Labyrinthula* sp. An independent t-test was conducted for both experimental data sets to determine if there was a difference in post-infection EQY values between the high salinity/high temperature and high salinity/high temperature/control treatment groups.

For both experiments (stressors maintained and recovery simulation) the pre- and post-infection EQY values for the groups formed by ambient and high salinity and ambient and high temperature were not normally distributed and could not be transformed. Therefore, a Wilcoxon Signed Ranks test was performed to determine if

there were differences between pre- and post-infection EQY values among ambient and high salinities and ambient and high temperatures groups.

In the experiment where stressors were maintained, the dependent variable, post-infection EQY, was not normally distributed for the groups formed by salinity and temperature so the data were transformed using an arcsin transformation. A two-way ANOVA was then conducted to test the effect of salinity and temperature on post-infection EQY values associated with infection from *Labyrinthula* sp. In the recovery simulation, data were not normally distributed for the post-infection EQY groups formed by salinity and temperature and could not be transformed. Therefore a Kruskal-Wallis test was utilized.

An independent t-test was also conducted to compare the lesion sizes between experiment 1a (stressors maintained throughout) and experiment 1b (recovery simulation).

Sulfide + Hypoxia (Stressors Maintained)

The groups formed by sulfide and oxygen were not normally distributed and could not be transformed, therefore a Kruskal-Wallis test was utilized to determine differences in lesion size among main effects.

The control and sulfide + hypoxia groups were distributed normally for post-infection EQY values, therefore, an independent t-test was conducted to determine if differences existed between the two groups. The groups formed by 0 mM and 6 mM sulfide were parametric. A dependent t-test was utilized to determine if there were

significant differences between pre- and post-infection EQY values and an ANOVA was used to determine differences between post-infection EQY values.

The groups formed by hypoxia and normoxia were not normally distributed and could not be transformed, therefore, a Wilcoxon Signed Ranks test was utilized to determine differences between the pre- and post-infection EQY values. A Kruskal-Wallis test was utilized in determining differences between post-infection EQY values for the effect of hypoxia.

Salinity + Temperature + Sulfide + Hypoxia (Stressors Maintained and Recovery Simulation)

For both the recovery simulation and when the stressors were maintained, the experiment was split into two groups due to size constraints and data were square root transformed to meet the assumptions of the statistical test. A t-test was utilized to determine if differences existed between the two groups. When differences existed, a randomized block design was utilized to statistically control for time as it was not of primary relevance to the research.

For lesion analysis in both experiments, data were square root transformed to meet the assumptions of the test and a randomized block ANOVA (4-way) was conducted with time as a nuisance factor when applicable. When interactions were present, ANOVAs were utilized to determine individual effects.

In both experiments, none of the groups formed by the main effects were parametric for either pre-infection or post-infection EQY values and they could not be transformed. Therefore, nonparametric tests were utilized with all EQY data.

Differences in post-infection EQY values between the control and the elevated salinity + elevated temperature + sulfide + hypoxic group were analyzed using a Kruskal-Wallis test. Wilcoxon Signed Ranks tests were conducted to determine if statistically significant differences were present between pre-infection and post-infection EQY values for each main effect. Kruskal-Wallis tests were also performed to determine if there were statistically significant differences in post-infection EQY values among the main effects and to determine if there were differences in lesion sizes between the experiment 3a (stressors maintained throughout) and experiment 3b (recovery simulation).

In Vitro Labyrinthula sp. Growth Assay

A two-way ANOVA was conducted to test the effects of salinity and temperature on the growth rate (measure as colony area) of *Labyrinthula* sp.

2.3 Results

Elevated Salinity and Temperature on T. testudinum- Sustained Stress Conditions

One replicate in the high salinity/high temperature treatment group was discarded due to mortality prior to infection with *Labyrinthula* sp.

Lesion Size: Salinity + Temperature (Stressors Maintained)

There was no significant interaction between salinity and temperature ($p = 0.903$), but the effect of salinity was shown to be statistically significant ($p = 0.029$) with ambient salinity groups possessing larger lesions (Figure 5). The effect of temperature was not significant ($p = 0.187$).

Effective Quantum Yield: Salinity + Temperature (Stressors Maintained)

There were no significant ($p = 0.065$) differences in post-infection EQY values between the experimental group and the control (Figure 6). Both salinity and temperature had statistically significant differences ($p < 0.07$) between pre- and post-EQY infection values (Figure 7). However, there were no significant interactions or main effects among post-infection EQY values.

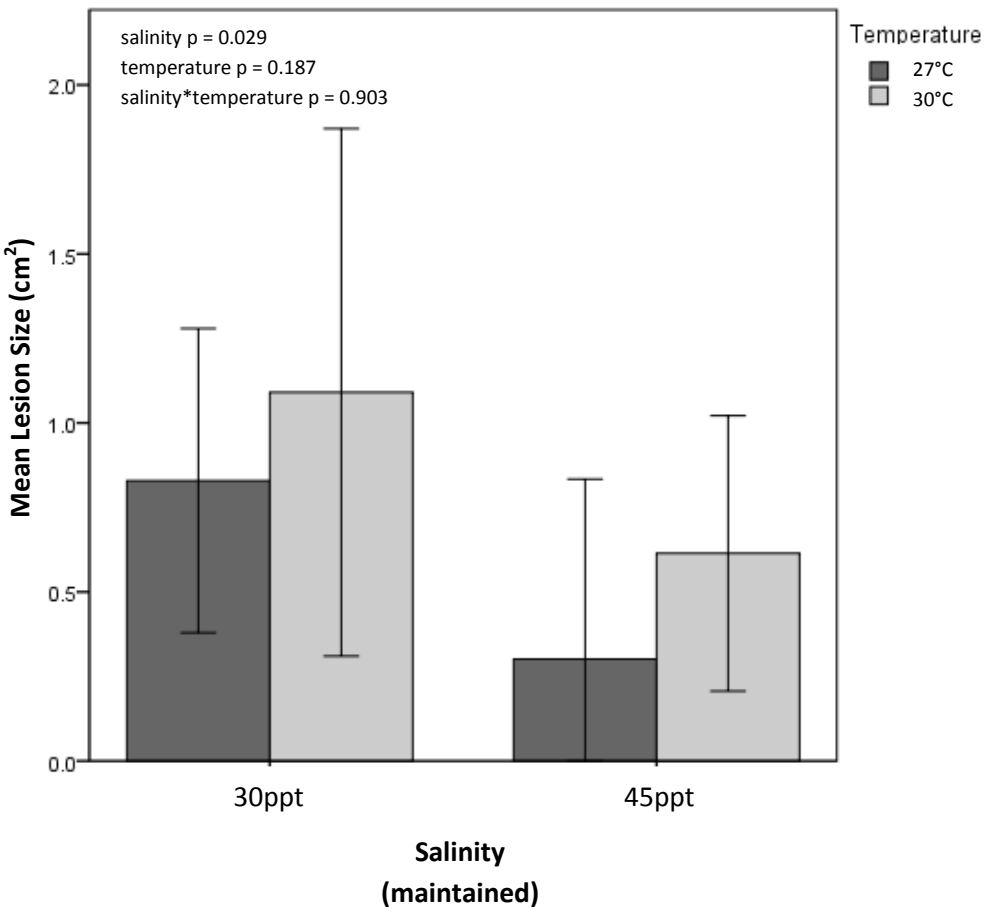

Figure 5 *Thalassia testudinum* blades infected with *Labyrinthula* sp. under ambient salinity groups had significantly larger lesions on average than groups incubated under high salinity conditions. There was no significant difference in lesion size due to temperature. Bars represent 95% CI.

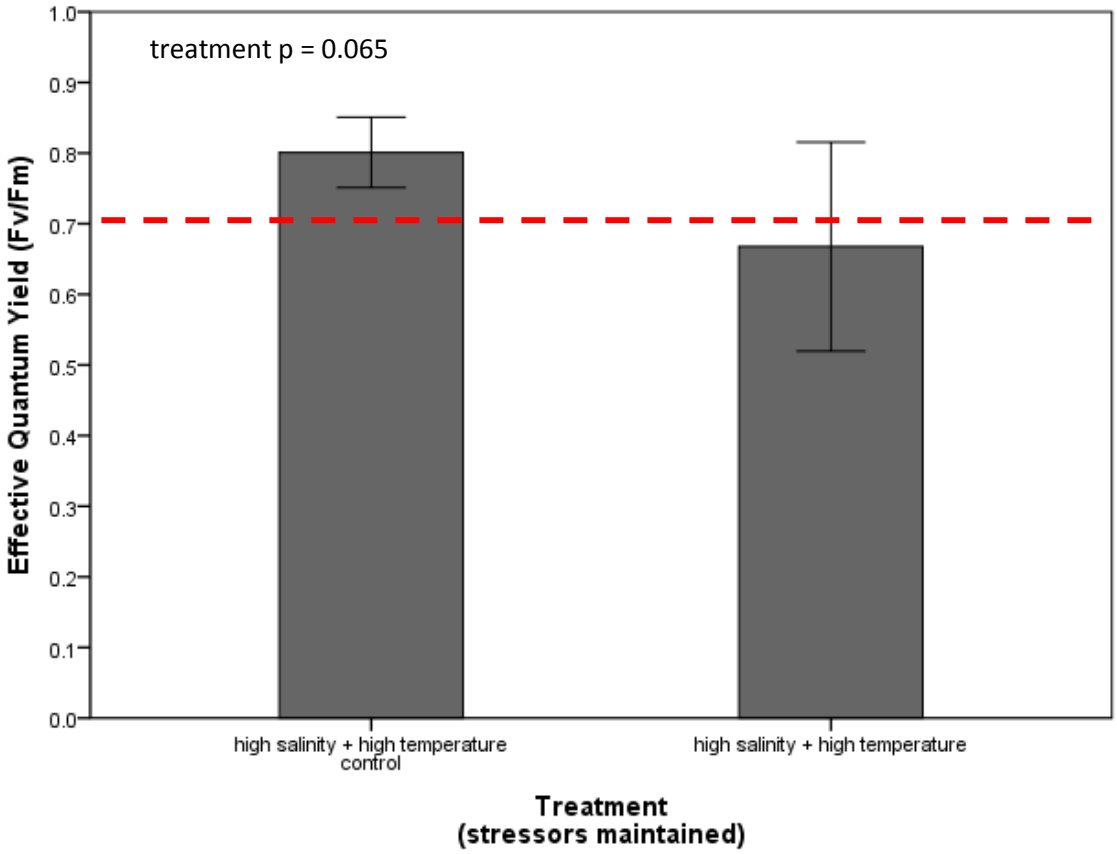

Figure 6 Post-infection EQY values between the high salinity + high temperature groups exposed to *Labyrinthula* sp. and the control group (no *Labyrinthula* sp.) exposed to the same stressors were not significantly different. Dotted line represents an effective quantum yield value of 0.700, a quantifiable indicator of stress among *T. testudinum*. Bars represent 95% CI.

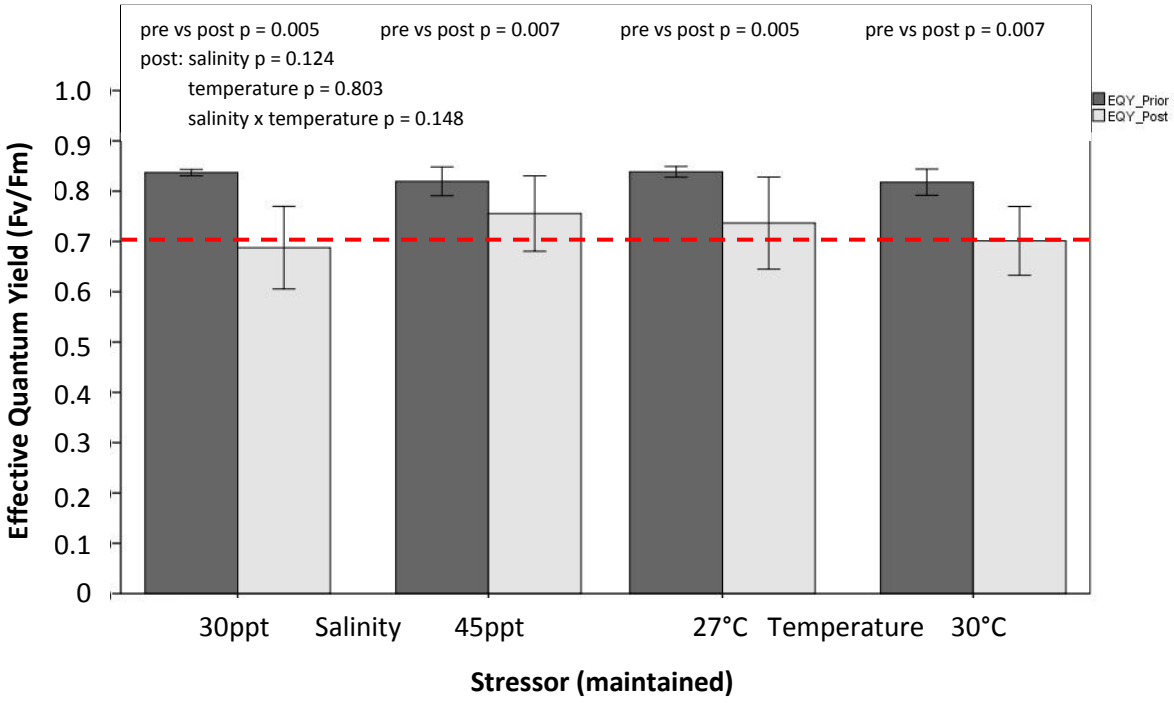

Figure 7 Pre-infection EQY values for the groups formed by ambient and high salinity and ambient and high temperature were significantly different from their corresponding post-infection EQY values. However, there were no significant differences in main effects among post-infection EQY values. Dotted line represents an effective quantum yield value of 0.700, a quantifiable indicator of stress among *T. testudinum*. Bars represent 95% CI.

Elevated Salinity and Temperature on T. testudinum- Recovery Simulation

Individual shoots of *T. testudinum* were incubated under various combinations of salinities and temperatures for one week after which they were returned to ambient conditions and infected with *Labyrinthula* sp.

Lesion Size: Salinity + Temperature (Recovery)

There was no significant interaction between salinity and temperature, and the effects of salinity and temperature were not significant (Figure 8).

Effective Quantum Yield: Salinity + Temperature (Recovery)

There were no statistically significant ($p = 0.386$) differences in post-infection EQY values between the control and the high salinity/high temperature groups (Figure 9). However, there were statistically significant differences among pre- and post-infection EQY values for both ambient ($p = 0.005$) and high ($p = 0.012$) salinities. Additionally, ambient salinity groups had post-infection EQY values below 0.700. There were also were statistically significant differences among pre- and post-infection EQY values for both ambient ($p = 0.005$) and high ($p = 0.012$) temperatures. Both high and ambient temperature groups had post-infection EQY values below 0.700 (Figure 10). There were no statistically significant differences among post-infection EQY values for the groups formed by either salinity ($p = 0.450$) or temperature ($p = 0.650$).

There was a statistically significant difference ($p < 0.001$) in lesion size between the experiment where stressors were maintained throughout and the recovery simulation with the recovery group exhibiting larger lesions (Figure 11).

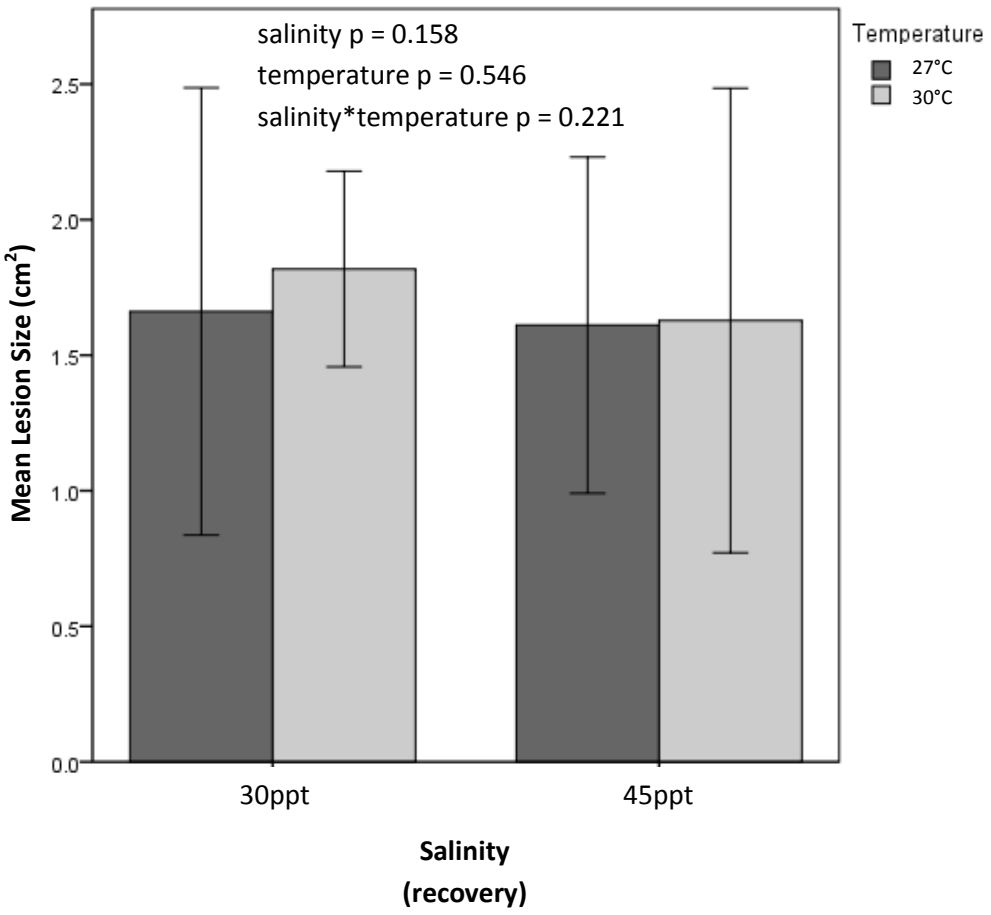

Figure 8 Lesion size for salinity and temperature under a recovery simulation (*Labyrinthula* sp. was not exposed to stressors). There were no differences in lesion size due to either main effect. Bars represent 95% CI.

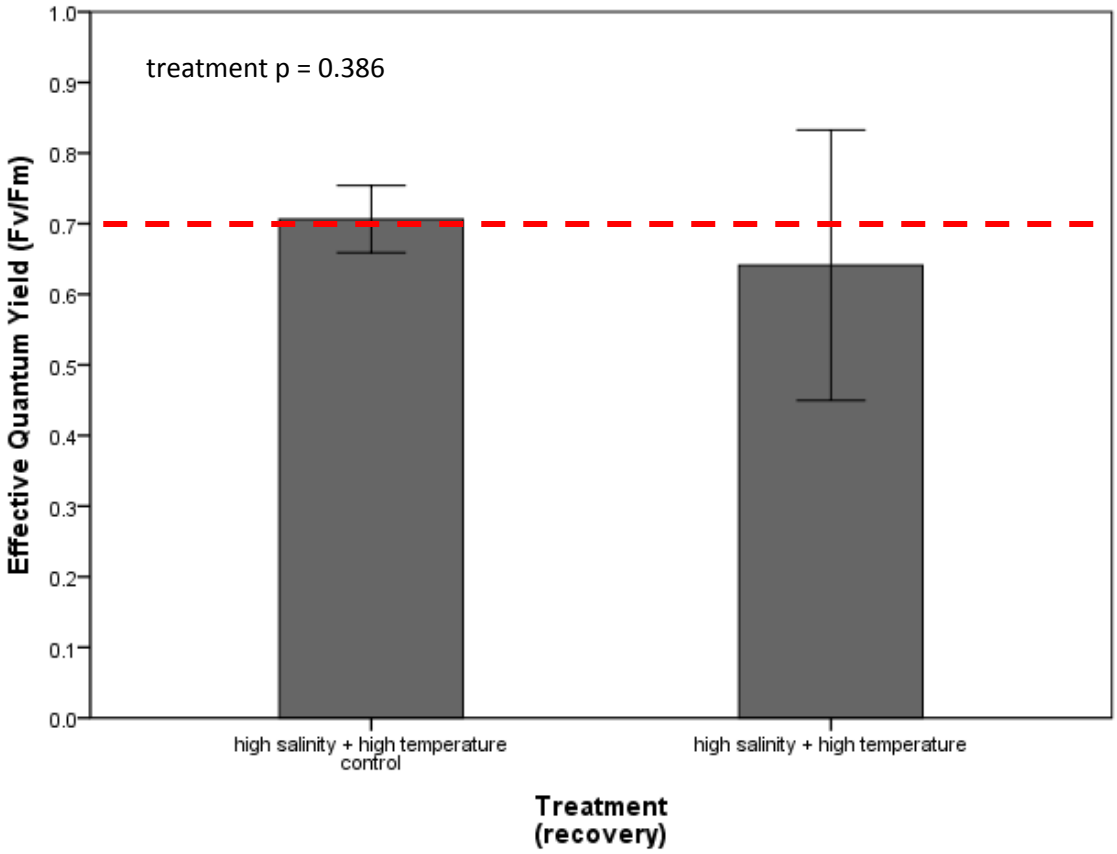

Figure 9 Post-infection EQY values between the control group (no *Labyrinthula* sp.) and the group exposed to the same stressors under a recovery simulation were not significantly different. Dotted line represents an effective quantum yield value of 0.700, a quantifiable indicator of stress among *T. testudinum*. Bars represent 95% CI.

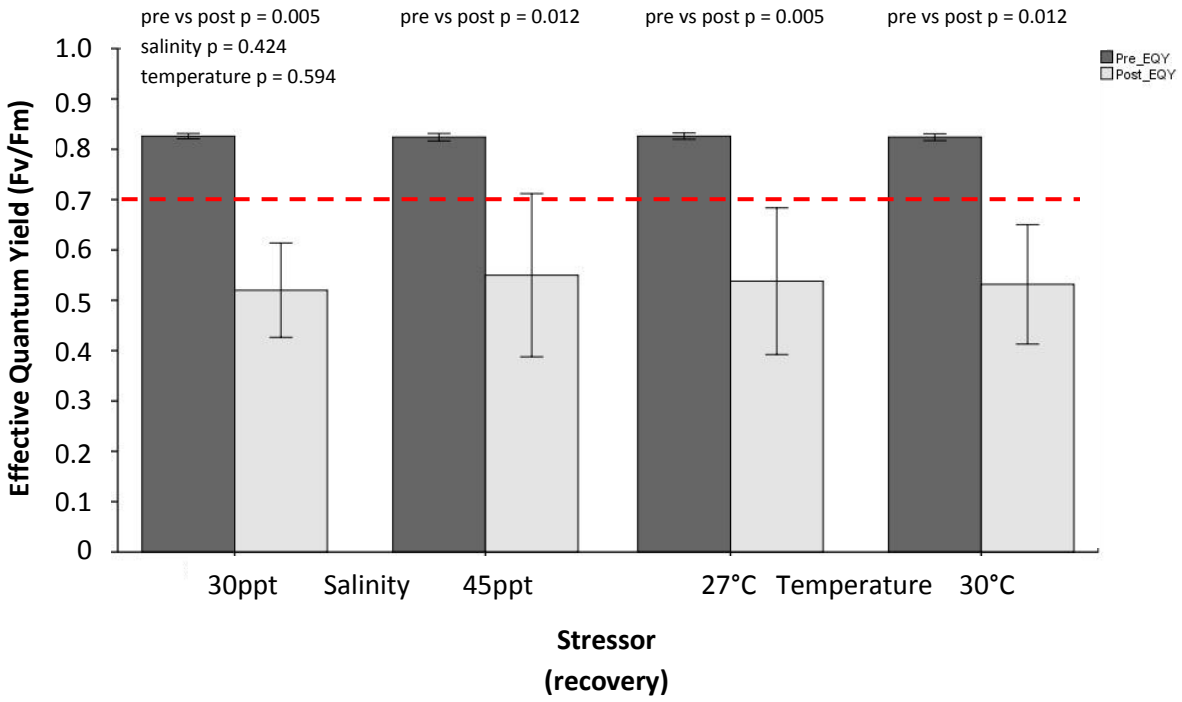

Figure 10 Comparison of pre- and post-infection EQY for salinity and temperature under a recovery simulation. All main effects had significantly lower EQY values after infection with *Labyrinthula* sp. All main effects also had mean post-infection EQY values below 0.700. However, here were no statistically significant differences in post-infection EQY values for the groups formed by either salinity or temperature. Dotted line represents an effective quantum yield value of 0.700, a quantifiable indicator of stress among *T. testudinum*. Bars represent 95% CI.

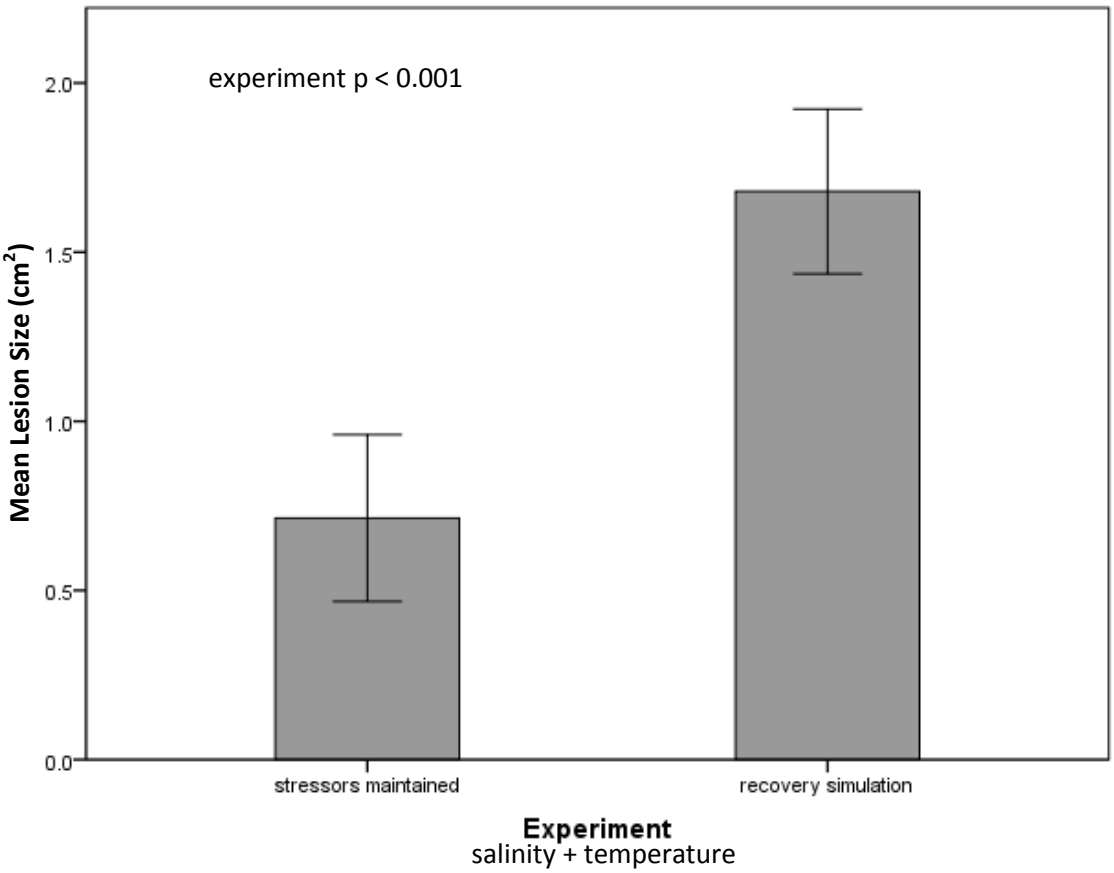

Figure 11 Comparison of mean lesion size between the sustained stress condition and the recovery simulation. Lesions were significantly smaller in the experiment where stressors were maintained throughout. Bars represent 95% CI.

Night-time Hypoxia and Sulfide on T. testudinum- Sustained Stress Conditions

Individual shoots of *T. testudinum* were incubated under various combinations of dissolved water column oxygen and sulfide for one week after which they were infected with *Labyrinthula* sp. and continued incubation under stressor conditions. Lesion size and photosynthetic efficiency were quantified and used as a measure of proxy for seagrass health.

Lesion Size: Sulfide + Hypoxia (Stressors Maintained)

The groups exposed to sulfide had significantly ($p = 0.034$) larger lesions than those not exposed sulfide (Figure 12). There were no significant differences ($p = 0.705$) in lesion size between hypoxic and normoxic groups.

Effective Quantum Yield: Sulfide + Hypoxia (Stressors Maintained)

There were no statistically significant ($p = 0.810$) differences in post-infection EQY values between the sulfide + hypoxia control group (no *Labyrinthula* sp.) and the sulfide + hypoxia groups exposed to *Labyrinthula* sp. (Figure 13). There were also no statistically significant differences (0mM $p = 0.101$; 6mM $p = 0.113$) in pre-infection and post-infection EQY values (Figure 14). There were no statistically significant ($p = 0.578$) differences between post-infection EQY values between groups exposed to sulfide and those not exposed to sulfide.

A statistically significant ($p = 0.011$) difference was found between the pre-infection and post-infection EQY values for the hypoxic group (Figure 14). However,

there were no statistically significant ($p = 0.902$) differences in post-infection EQY values due to hypoxia.

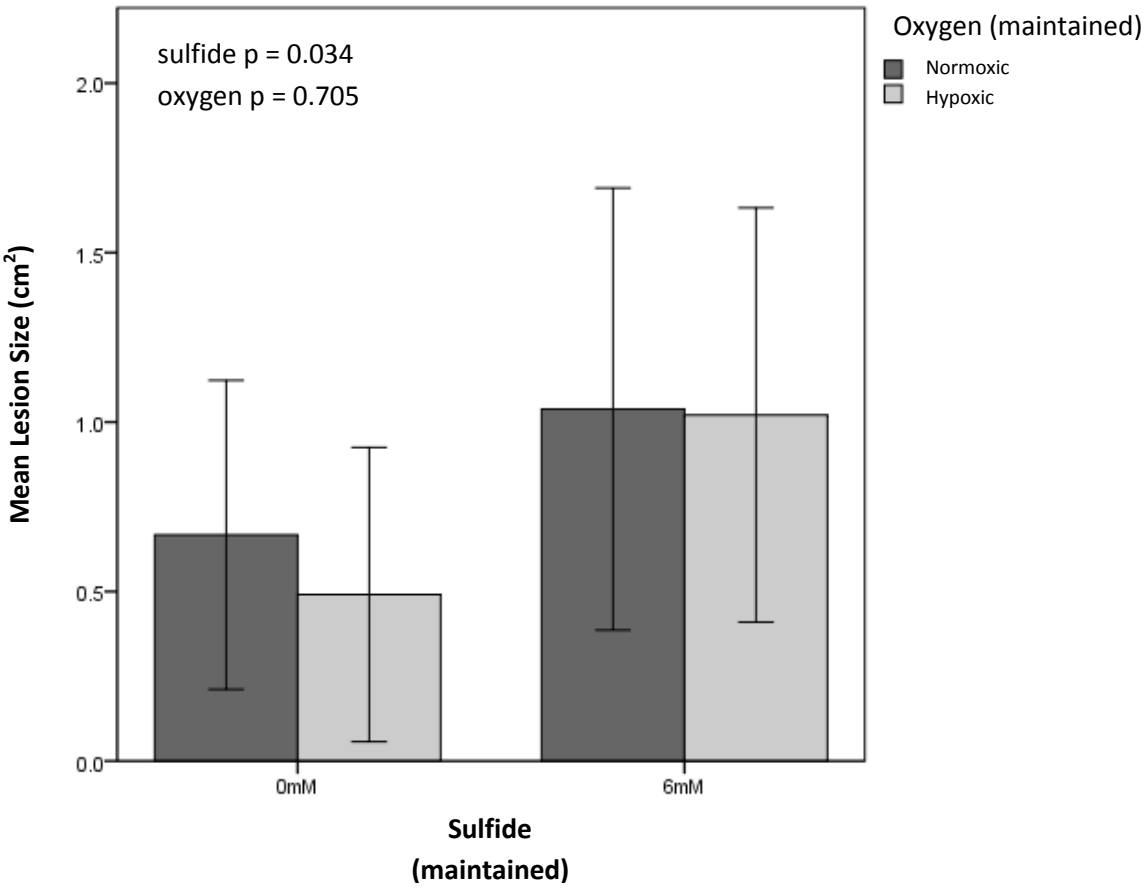

Figure 12 Comparison of lesion size due to the effects of sulfide and oxygen. Seagrasses exposed to 6mM of sulfide had significantly larger lesions than groups not exposed to sulfide. There were no statistically significant differences in lesion size due to hypoxia. Bars represent 95% CI.

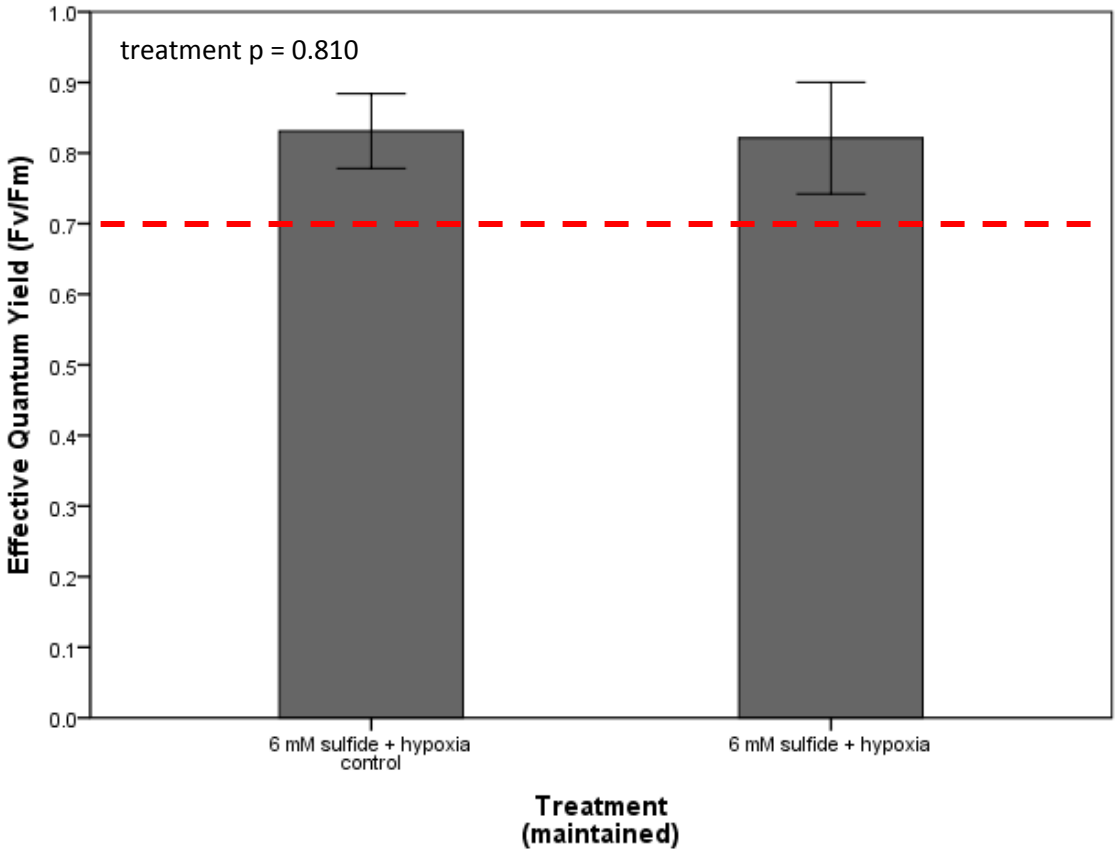

Figure 13 Comparison of post-infection EQY values between the control group (no *Labyrinthula* sp.) and the group exposed to similar stressors. There were no significant differences between the groups. Dotted line represents an effective quantum yield value of 0.700, a quantifiable indicator of stress among *T. testudinum*. Bars represent 95% CI.

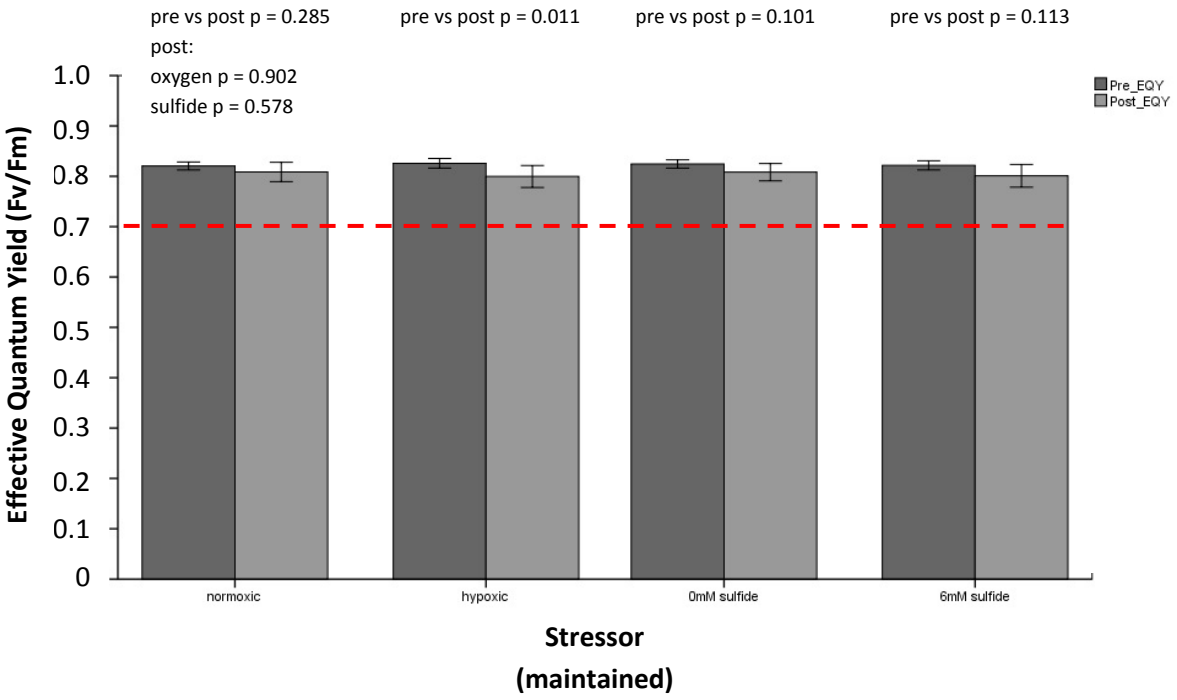

Figure 14 Pre- vs post-infection EQY values for the effects of oxygen and sulfide. There were no significant differences between pre- and post-infection EQY values among sulfide treatments. However, hypoxic groups possessed significantly (p = .011) lower post-infection EQY values when compared to pre-infection EQY values. There were no differences in post-infection EQY values among the groups formed by sulfide or oxygen treatments. Dotted line represents an effective quantum yield value of 0.700, a quantifiable indicator of stress among *T. testudinum*. Bars represent 95% CI.

Elevated Salinity + Elevated Temperature + Night-time Hypoxia + Sulfide on T. testudinum- Sustained Stress Conditions

T. testudinum were exposed to various combinations of salinity, temperature, dissolved oxygen and sulfide concentrations in a full factorial experimental design. Experimental conditions were maintained throughout the duration of the experiment.

Lesion Size: Salinity + Temperature + Hypoxia + Sulfide (Stressors Maintained)

The effect of temperature ($p = 0.007$) was significant with ambient temperatures resulting in larger lesion sizes (Figure 15). There was also a significant interaction between salinity and sulfide ($p = 0.018$). Salinity was found to be statistically significant ($p = 0.000$), but the effect of sulfide was not significant ($p = 0.465$) indicating that the effects of salinity were not the same at all levels of sulfide. With ambient salinity, high sulfide concentrations resulted in larger lesion sizes (Figure 16).

Effective Quantum Yield: Salinity + Temperature + Hypoxia + Sulfide (Stressors Maintained)

There were no statistically significant ($p = 0.465$) differences in post-infection EQY values between the control and the elevated salinity + elevated temperature + sulfide + hypoxia groups (Figure 17).

There were statistically significant differences between pre- and post-infection EQY values for the following groups (Figure 18): ambient salinity had lower post-infection EQY values ($p = 0.001$); ambient temperature had lower post-infection EQY

values ($p = 0.031$); the 0mM sulfide group had lower post-infection EQY values ($p = 0.036$); hypoxic groups had lower post-infection EQY values ($p = 0.005$).

Temperature was the only significant effect ($p = 0.025$) among post-infection EQY values. High temperature resulted in lower post-infection EQY values (Figure 18). There were no statistically significant differences for salinity ($p = 0.065$), sulfide ($p = 0.542$) or oxygen ($p = 0.322$).

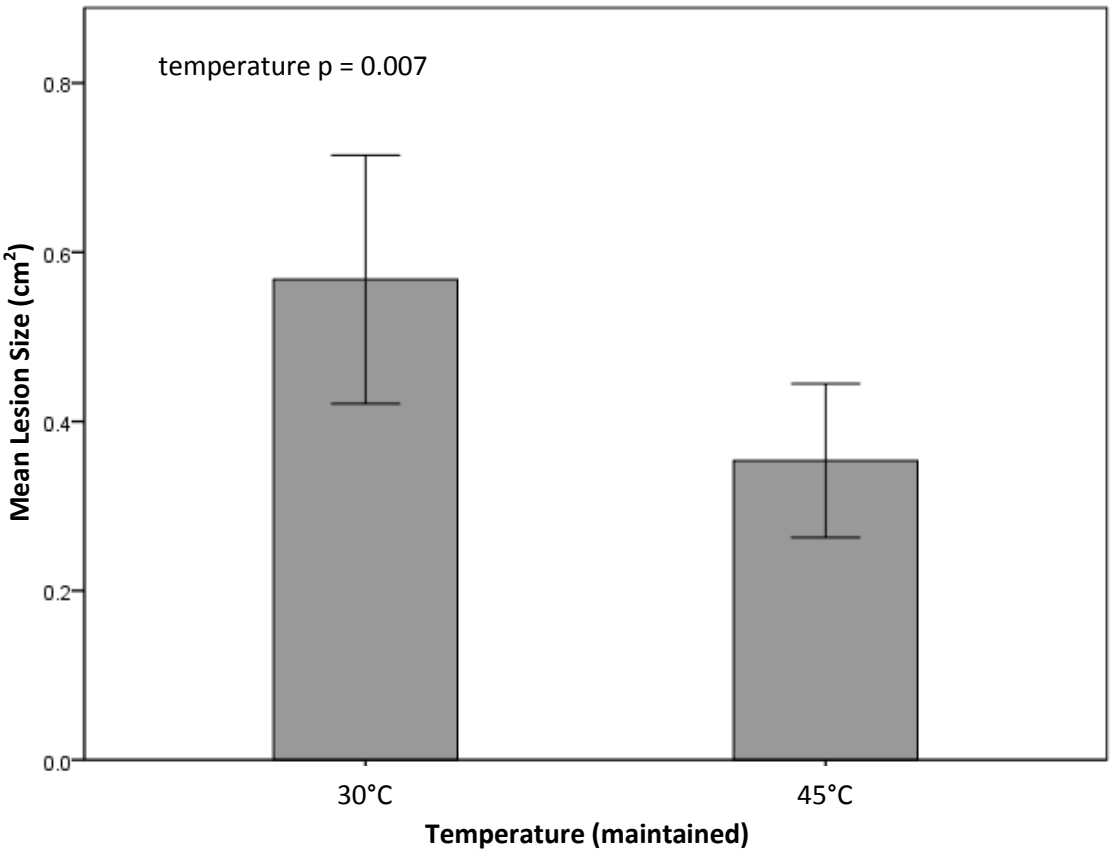

Figure 15 Comparison of lesion size for temperature groups when seagrasses were exposed to a combination of salinity, temperature, sulfide and oxygen. Stressors were sustained throughout the experiment. Ambient temperature groups had significantly larger lesions than high temperature groups. Bars represent 95% CI.

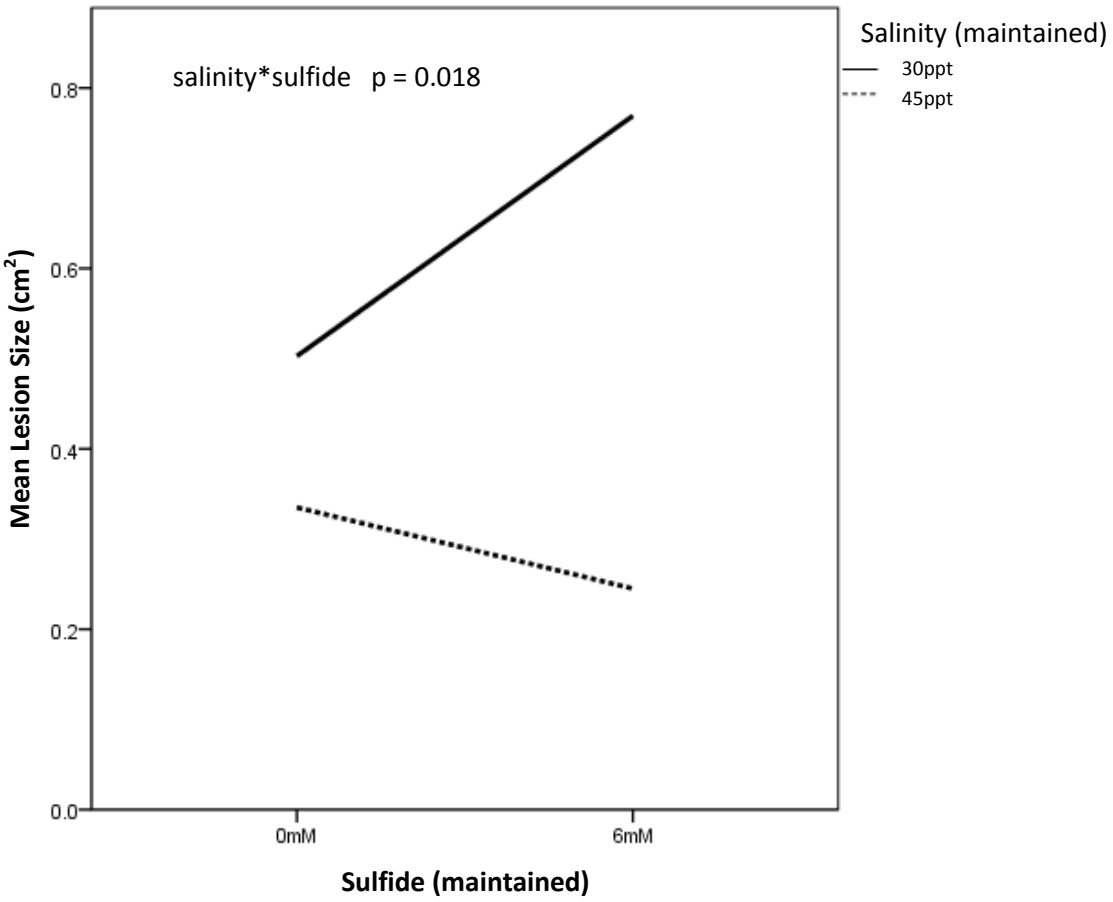

Figure 16 An interaction between salinity and sulfide indicated that at ambient salinity, high sulfide results in larger lesions

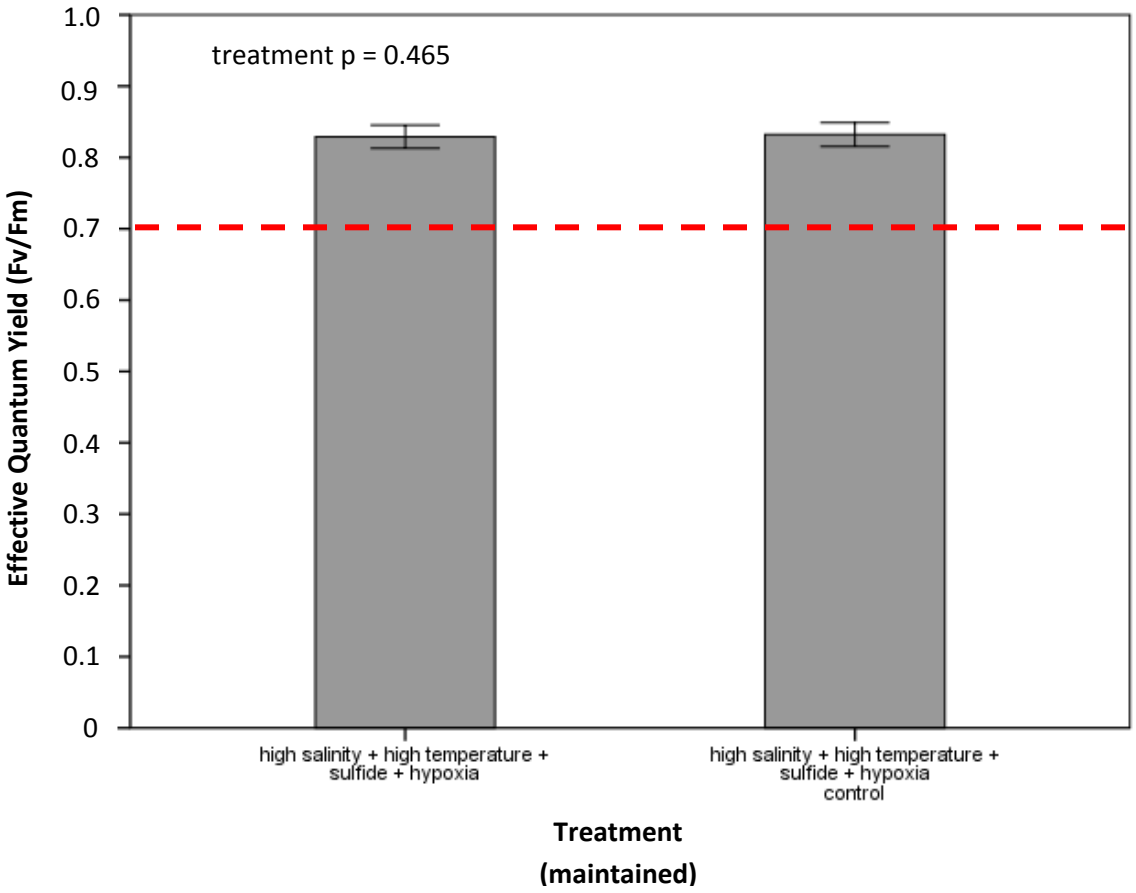

Figure 17 Post-infection EQY values for the control (no *Labyrinthula* sp.) and the group exposed to similar stressors. There was no significant difference between the groups. Dotted line represents an effective quantum yield value of 0.700, a quantifiable indicator of stress among *T. testudinum*. Bars represent 95% CI.

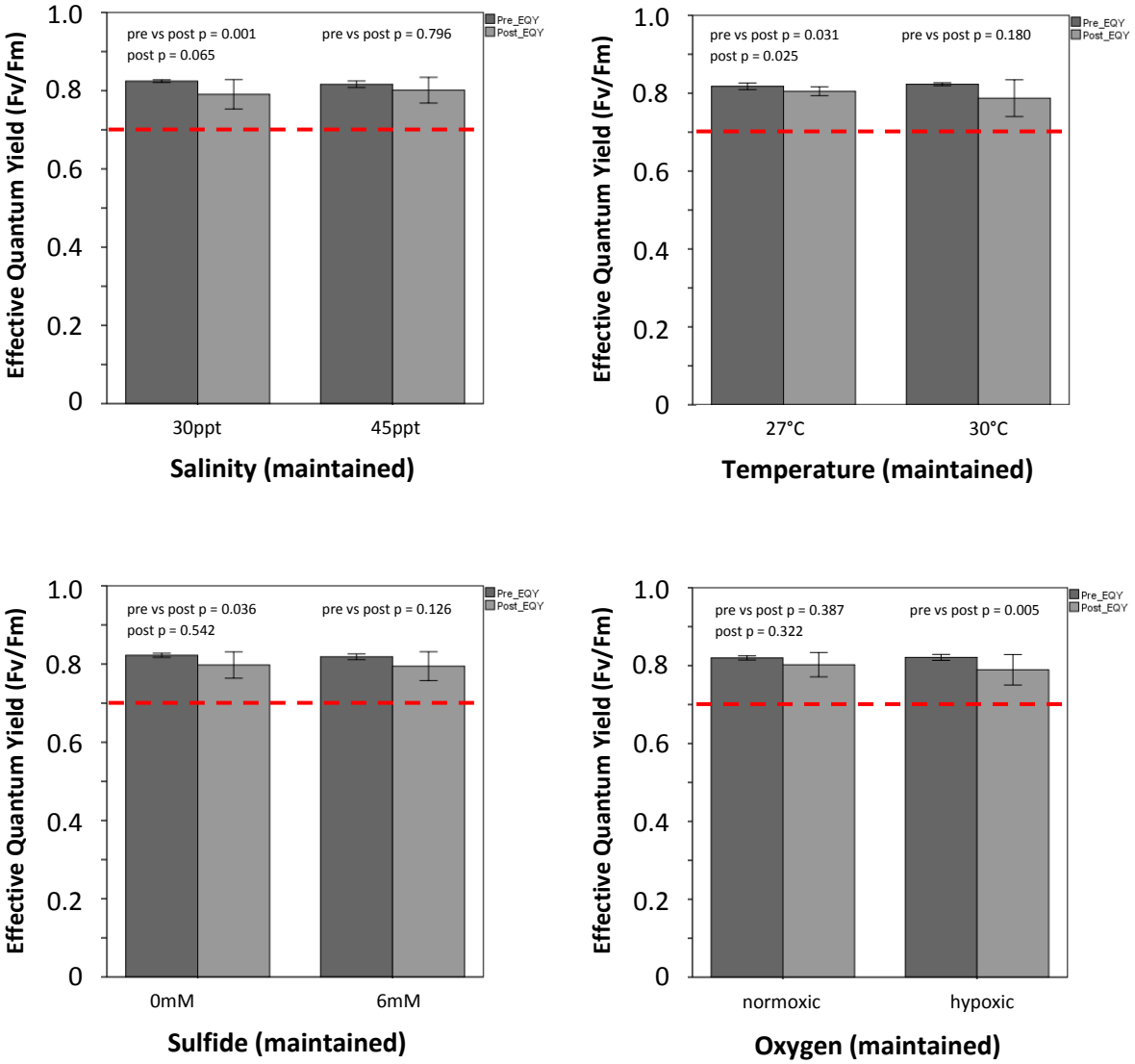

Figure 18 Comparison of pre- and post-infection EQY values for the effects of salinity, temperature, sulfide and oxygen under sustained stress conditions. There were significant differences between pre- and post-infection EQY values for ambient salinity, ambient temperature, 0 mM sulfide and hypoxia. The only significant difference between post-infection EQY values was found among temperature groups, with elevated temperatures resulting in lower post-infection EQY values. Dotted line represents an effective quantum yield value of 0.700, a quantifiable indicator of stress among *T. testudinum*. Bars represent 95% CI.

Elevated Salinity + Elevated Temperature + Night-time Hypoxia + Sulfide on T. testudinum- Recovery Simulation

T. testudinum were exposed to various combinations of salinity, temperature, dissolved oxygen and sulfide concentrations in a full factorial experimental design that simulated a recovery scenario. Experimental conditions were maintained for one week after which they were returned to ambient conditions and infected with *Labyrinthula* sp. so that *Labyrinthula* sp. was not exposed to stressors.

The experiment was split into two groups due to size constraints, however, there were no differences between the two groups.

Lesion Size: Salinity + Temperature + Hypoxia + Sulfide (Recovery)

There was a significant ($p = 0.039$) interaction among salinity, temperature, sulfide and oxygen. Therefore, separate three-way ANOVAs were utilized for each combination of main effects. None of the three-way ANOVAs revealed any significant interactions, but salinity was found to be significant ($p = 0.003$). Regardless of temperature, sulfide or oxygen, ambient salinity groups demonstrated larger lesion sizes (Figures 19, 20, 21, 22).

Effective Quantum Yield: Salinity + Temperature + Hypoxia + Sulfide (Recovery)

There was no statistically significant ($p = 0.465$) difference in post-infection EQY values between the control and the elevated salinity + elevated temperature + sulfide + hypoxia groups (Figure 23).

Wilcoxon Signed Ranks determined there were statistically significant ($p < 0.001$ for all) differences between pre- and post-infection EQY values for all levels of each of the main effects. In all cases, post-infection EQY values were significantly lower (Figure 24).

Kruskal-Wallis tests were performed to determine if there were statistically significant differences in post-infection EQY values among the main effects. Salinity was the only significant effect ($p = 0.039$) with ambient salinity resulting in lower post-infection EQY values (Figure 24). There were no statistically significant differences for temperature ($p = 0.290$), sulfide ($p = 0.499$) or oxygen ($p = 0.641$).

A Kruskal-Wallis test was also conducted to compare the lesion sizes between experiment 3a (stressors maintained throughout) and experiment 3b (recovery simulation). There was a statistically significant difference ($p < 0.001$) in lesion size between the two experiments with the recovery experiment exhibiting larger lesions (Figure 25).

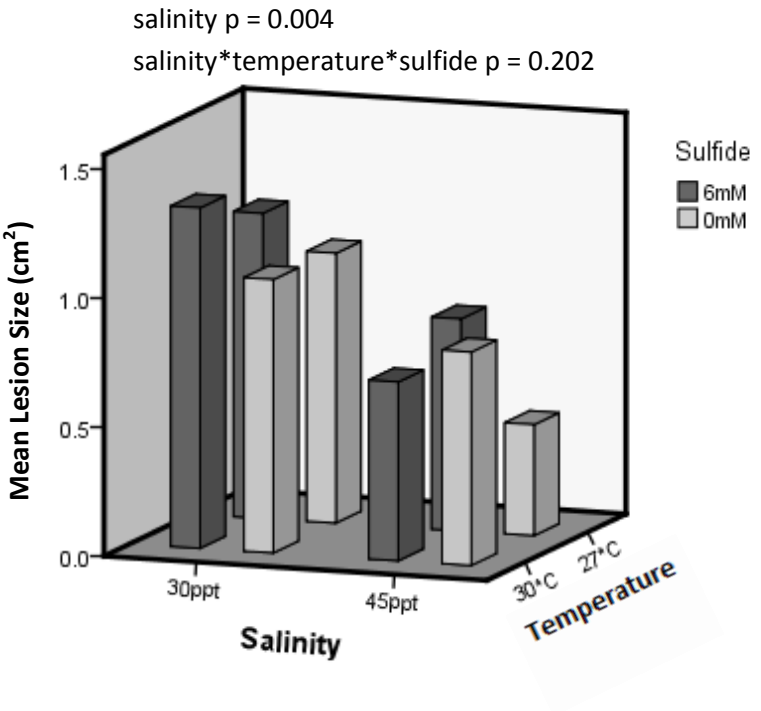

Figure 19 Comparison of salinity, temperature and sulfide due to the interaction of all main effects under a recovery simulation. Seagrasses in ambient salinity groups had significantly larger lesions regardless of temperature or sulfide.

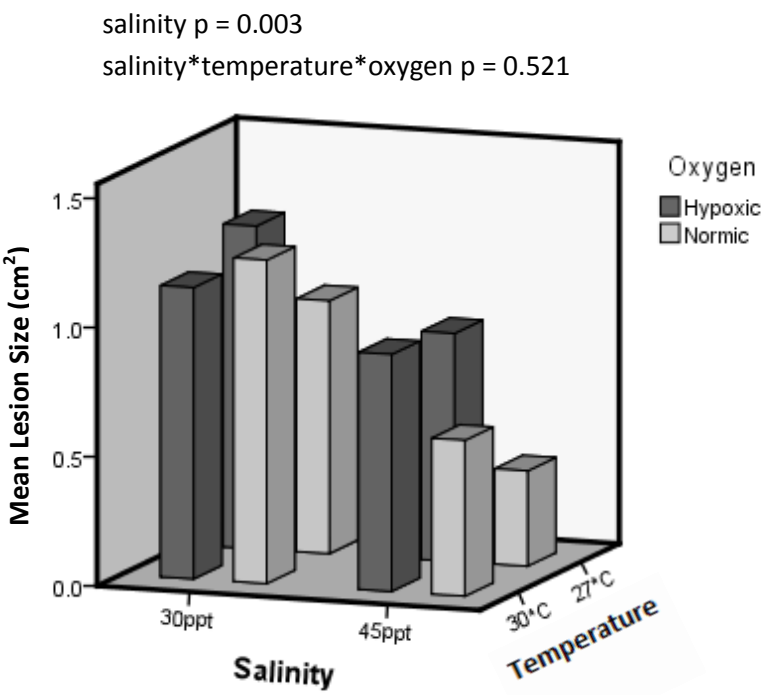

Figure 20 Comparison of salinity, temperature and oxygen due to the interaction of all main effects under a recovery simulation. Seagrasses in ambient salinity groups had significantly larger lesions regardless of temperature or sulfide.

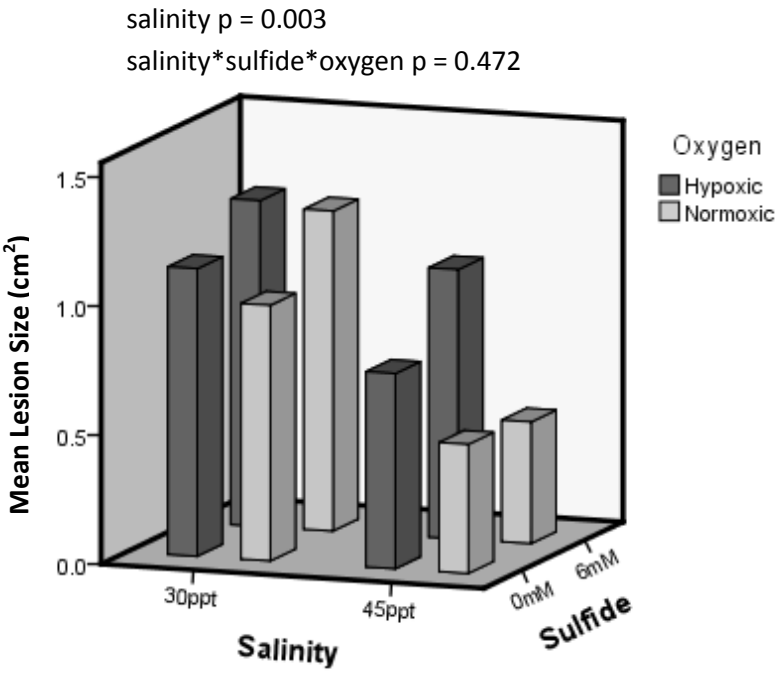

Figure 21 Comparison of salinity, sulfide and oxygen due to the interaction of all main effects under a recovery simulation. Seagrasses in ambient salinity groups had significantly larger lesions regardless of temperature or sulfide.

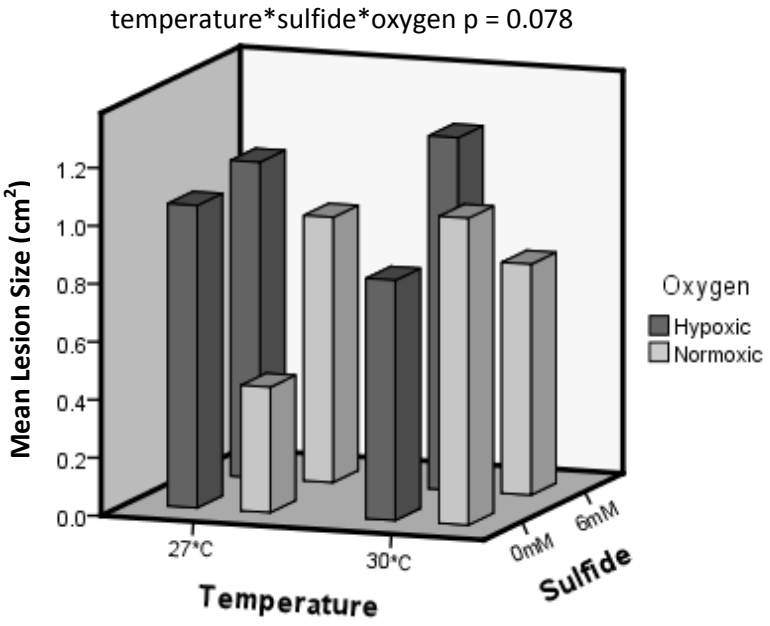

Figure 22 Comparison of temperature, sulfide and oxygen due to the interaction of all main effects under a recovery simulation. There was no significance for any of these effects.

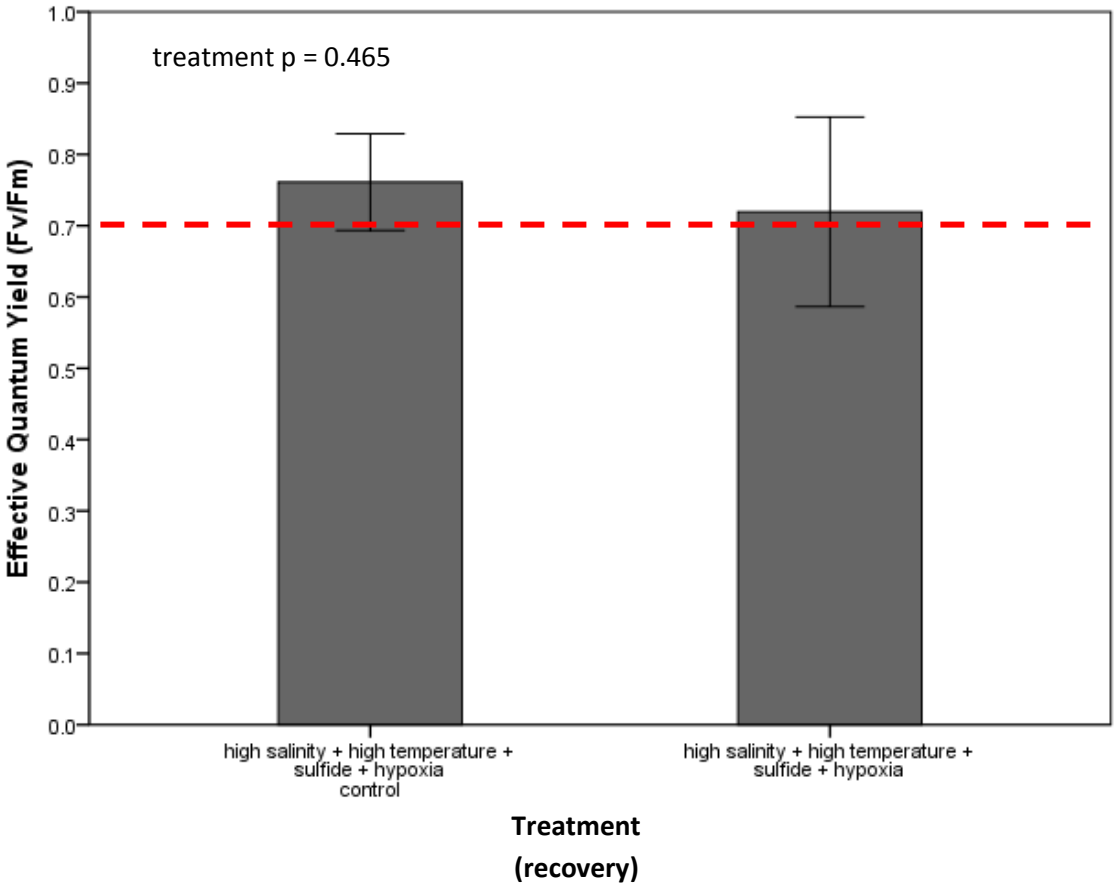

Figure 23 Post-infection EQY values for the control and stress group under a recovery simulation. There were no statistically significant differences between the control (no *Labyrinthula* sp.) and the group with similar stressors. Dotted line represents an effective quantum yield value of 0.700, a quantifiable indicator of stress among *T. testudinum*. Bars represent 95% CI.

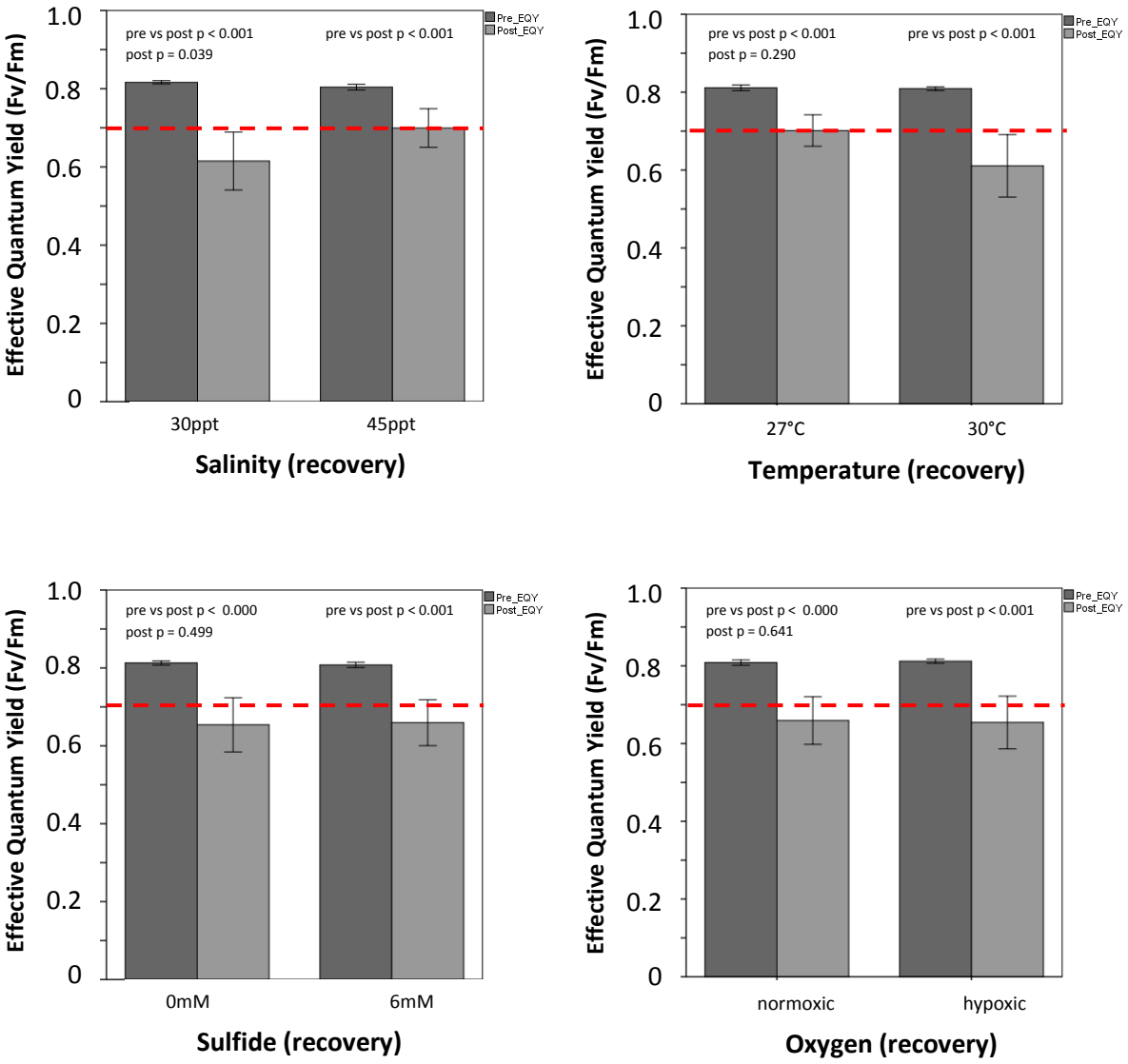

Figure 24 Comparison of the pre- and post-infection EQY values for the effects of salinity, temperature, sulfide and oxygen under a recovery simulation. There were significant differences between pre- and post-infection EQY values for all main effects at all levels. However, the only significant difference among post-infection EQY values between the salinity groups with ambient salinity resulting in lower post-infection EQY values. Dotted line represents an effective quantum yield value of 0.700, a quantifiable indicator of stress among *T. testudinum*. Bars represent 95% CI.

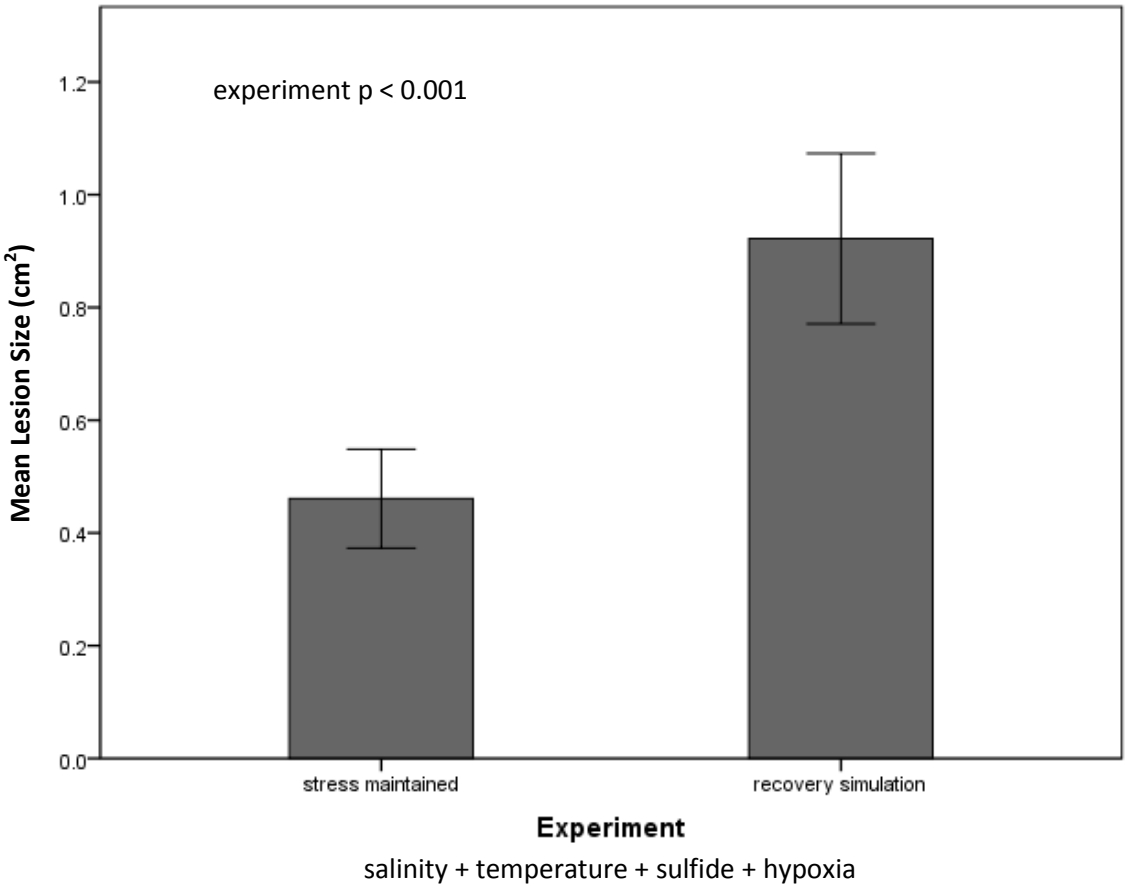

Figure 25 Comparison of lesion size for the sustained stressors experiment and recovery simulation. Lesion size was significantly smaller in the experiment where stressors were maintained throughout. Bars represent 95% CI.

In Vitro Labyrinthula sp. Growth Assay

There was no statistically significant interaction between salinity and temperature ($p = 0.266$). However, the effect of salinity was shown to be statistically significant ($p < 0.001$) (Figure 26).

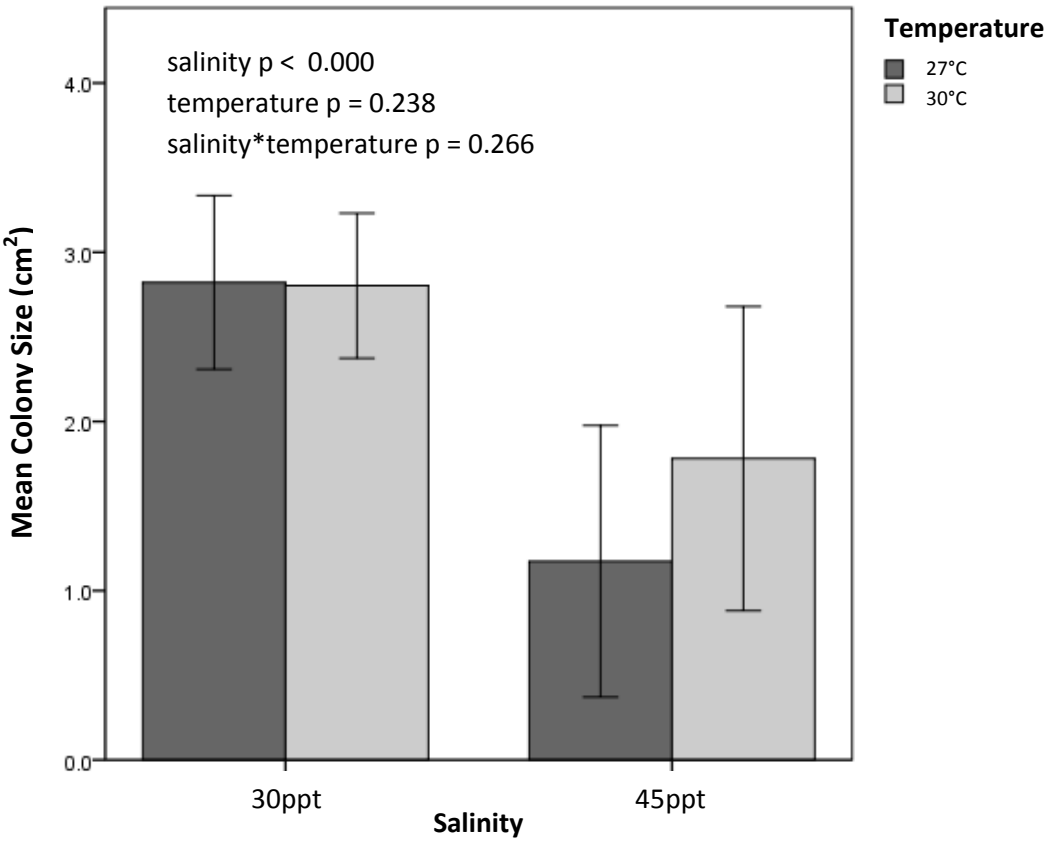

Figure 26 Colony size for *Labyrinthula* sp. incubated under varying salinities and temperatures. The effect of salinity was significant on the growth of *Labyrinthula* sp. Ambient salinity groups had significantly larger colony area compared to high salinity groups. Temperature did not have a significant effect. Bars represent 95% CI.

2.4 Discussion

Seagrasses are declining worldwide (Bergmann *et al.* 2010; Bull *et al.* 2012; Duarte 2002; Orth *et al.* 2006; Papenbrock 2012; Touchette 2007). A major contributor to the loss of seagrass beds is wasting disease. Although the causal agent of wasting disease has been identified as a Labyrithulid protist (Bull *et al.* 2012; Muehlstein *et al.* 1991; Ralph and Short 2002; Short *et al.* 1987), the role of abiotic stressors in relation to susceptibility and infection is still unclear (Orth *et al.* 2006). Previous studies have suggested that environmental stressors contribute to increased prevalence of wasting disease (Blakesley *et al.* 2001), therefore it was hypothesized that abiotic stressors would effectively weaken seagrasses, thus making them more susceptible to *Labyrinthula* sp. infection. Furthermore, it was proposed that multiple abiotic stressors would result in a cumulative effect as evidenced by increased lesion size and decreased photosynthetic efficiency. However, the results of this study indicate that the interactions among seagrasses, *Labyrinthula* sp. and the environment are not as clear-cut and linear as previously thought.

Although salinity and temperature combined did not have an additive or synergistic effect on seagrass health, the results of this study do suggest that the *Labyrinthula* sp. was affected by environmental stressors. When stress conditions were maintained throughout the trial period (i.e. *Labyrinthula* sp. was also exposed to elevated salinity and temperature) the ambient salinity groups had significantly larger lesions. However, under a recovery simulation (i.e. *Labyrinthula* sp. was not exposed to stressors) there were no differences in lesion size among treatment groups. Additionally, when lesion sizes between the two experimental scenarios were compared, there was a

significant difference in lesion size with the recovery group exhibiting lesions up to 250% larger. These results demonstrate that the pathogenic causal agent of wasting disease was also susceptible to environmental stressors, mainly salinity. Therefore, lesion size was not only influenced by the health of the seagrass, but was indicative of the health of the pathogen.

In a similar study, Trevathan *et al.* (2011) examined the effects of hypersalinity and *Labyrinthula* sp. infection. Similarly, elevated salinity groups exhibited significantly smaller lesions indicating *Labyrinthula* sp.'s sensitivity to hypersaline conditions. Other studies also corroborate that salinity is a driving component of wasting disease and *Labyrinthula* sp. health. Martin *et al.* (2009) grew *Labyrinthula* sp. colonies in liquid media under varying salinities. Colony size was significantly reduced at a salinity of 50 ppt. In a field survey of Florida Bay, salinity was correlated with severity of wasting disease (Blakesley *et al.* 2001). It has been proposed that salinity negatively impacts the *Labyrinthula* spp.'s ectoplasmic network, thus affecting their ability to adhere to host (i.e. seagrass) tissue (Trevathan *et al.* 2011).

Despite that lesion size did not increase with multiple abiotic stressors, it can be concluded that the presence of *Labyrinthula* sp. did have a negative effect on the overall health of the seagrass. Pre-infection measurements of EQY were significantly higher than post-infection measurements regardless of the experimental conditions. Interestingly, under recovery simulations (regardless of stressors), not only were the post-infection EQY values significantly lower than pre-infection values, but the mean post-infection values were below 0.700, a quantifiable indicator of stress (Björkman and Demmig, 1987; Ralph, 1999; Durako *et al.*, 2002 in Koch *et al.* 2007a). It can be

concluded that not only did the presence of *Labyrinthula* sp. decrease the EQY of the seagrasses, but the absence of stressors in the presence of *Labyrinthula* sp. resulted in a "healthier" pathogen that was essentially capable of inducing a greater stress response (i.e. lowered EQY value).

The discrepancy between lesion size and reduced EQY can be explained in a study conducted by Ralph and Short (2002). In this study, they concluded that the necrotic lesions were not the only effect of *Labyrinthula* spp. infection. Green tissue could still host pathogenic *Labyrinthula* spp. and greatly reduce photosynthetic efficiency. Therefore, "healthy-looking" seagrass may still have compromised photosynthetic efficiency regardless of its physical appearance. The same could be said for the present study. Although the significance of lesion size was not consistent among main effects, the presence of *Labyrinthula* sp. did reduce the mean photosynthetic efficiency below 0.700 in all recovery simulations for all main effects. However, there were no differences between the control groups and similar stressor groups that were actually exposed to *Labyrinthula* sp. These data suggest that the presence of *Labyrinthula* sp. was a driving factor in seagrass health. If the combination of any given stressor plus the presence of *Labyrinthula* sp. resulted in a stress response, the ambient groups (i.e. no stressors) would be expected to possess higher EQY values. However, this was not the case. Regardless of microcosm conditions, post EQY values dropped below 0.700 in the presence of *Labyrinthula* sp. Indeed, the effects of an environmental stressor on the health of seagrass may be negligible in comparison to the stress caused by the presence of *Labyrinthula* sp.

If *Labyrinthula* sp. alone reduces the health of seagrass, regardless of environmental conditions, then a significant difference in the post-infection EQY values between the control group (exposed to stressors, but not *Labyrinthula* sp.) and the corresponding experimental group (stressors + *Labyrinthula* sp.) would be expected. However, there were no significant differences between the stress and control groups. Another assumption was that the presence of *Labyrinthula* sp. alone was not enough to elicit a significant stress response, therefore, the presence of *Labyrinthula* sp. plus abiotic stressors must be responsible for a reduction in post-infection EQY values. However, if a specific stressor, or combination of stressors, plus the presence of *Labyrinthula* sp. reduced the photosynthetic efficiency of the seagrass, then one would not expect to see a reduction of photosynthetic efficiency in the ambient treatment groups. However, all treatments groups expressed a significant reduction in post-infection EQY values. Therefore, it can be concluded that *Labyrinthula* sp. and abiotic stressors reduce the health of seagrass, but not in additive fashion.

T. testudinum has demonstrated a remarkable ability to resist sulfide poisoning during short term exposure to below ground tissues due to its high photosynthetic capacity which enables it to oxygenate its rhizosphere, thus neutralizing toxic sulfides (Erskine and Koch 2000 in Koch and Erskine 2001). The results of this study demonstrated that sulfide exposure in combination with *Labyrinthula* sp. may affect *T. testudinum's* ability to resist infection. Because the presence of *Labyrinthula* sp. decreases the photosynthetic efficiency of seagrasses, they may be limited in their ability to oxygenate their rhizosphere and effectively respond to sulfide stress (Carslon *et al.* 1994). Therefore, seagrasses exposed to sulfide and *Labyrinthula* sp. demonstrated larger

lesions. Additionally, seagrasses have demonstrated the ability to absorb oxygen directly from the water column when photosynthesis wanes, thereby enabling the continued transport of oxygen to their roots and rhizomes (Borum *et al.* 2005). However, if the water column is hypoxic, oxygen would be limited, as would their ability to passively diffuse and transport it. Therefore, seagrass health would be negatively affected. Indeed, seagrasses exposed to hypoxic conditions had significantly lower post-infection EQY values indicating a stress response.

Previous studies have demonstrated that prolonged exposure to elevated temperatures and sulfide can have an additive effect on seagrass health. High temperatures result in increased respiratory demands, therefore limiting the amount of oxygen that can be transported to the below ground tissues and utilized in oxidizing toxic sulfide compounds (Carlson *et al.* 1994). However, in this study under recovery simulations, elevated temperature and sulfide did not demonstrate an additive effect in regards to lesion size. Salinity was the only significant effect with ambient salinity resulting in larger lesions. However, all stressors significantly lowered post-infection EQY values indicating that the presence of *Labyrinthula* sp. in combination with abiotic stressors does have negative effects on seagrass health.

Results of field and laboratory studies on wasting disease can be varied and enigmatic. As such, it is necessary to address some limitations of this study.

Genetic variability within seagrass populations and *Labyrinthula* sp. may lead to inconclusive results and inadequate conclusions of observed phenomenon. Recent studies have demonstrated high genetic diversity among both seagrass hosts and

pathogens. *Labyrinthula* spp. in marine systems have demonstrated surprisingly high genetic diversity (Collado-Mercado *et al.* 2010 in Bockelmann *et al.* 2012). Seagrasses, particularly *T. testudinum*, also exhibit high intra-population genetic diversity (van Dijk and van Tussenbroek 2010 in Bricker *et al.* 2011). Indeed, no one genotype is dominant within the populations studied. Such genetic diversity within a given population, among both the host and the pathogen, may help to explain why some seagrass beds are completely decimated while adjacent populations are untouched (Robblee *et al.* 1991).

Another consideration when studying complex interactions is methodological approach. Plowright *et al.* (2008) suggest that reductionist methods may not be of use in analyzing disease relationships. In these types of studies, large systems are broken down into smaller units and focused on individually. As such, complex interactions may be overlooked or undetected altogether. Additionally, experimental manipulation alone may not be sufficient when studying relationships such as those between hosts and pathogens. Interdisciplinary approaches may be of use when investigating these types of relationships and may be effective in elucidating the results of this study.

2.5 Conclusion

It can be concluded that 1) the interaction(s) among *T. testudinum*, *Labyrinthula* spp. and the environment are complicated and not as linear as previously thought, 2) environmental factors have a greater influence on the interactions of *Labyrinthula* spp. and *T. testudinum* than previously thought, especially in regards to *Labyrinthula* spp. health/virulence, and 3) in comparison to other stressors tested, salinity is a major environmental factor affecting *Labyrinthula* sp.'s ability to elicit a stress response and/or

necrotic lesion. The effects of environmental stressors on host-pathogen relationships can be diverse, possibly due to genetic variability of both the host and the pathogen and/or a result of reductionist methodologies that neglect the complexities of various biotic and abiotic interactions. Therefore, the individual stress thresholds for both the host and the pathogen need to be considered.

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Vita

Nichole Bishop was born She has always loved the outdoors and the biological sciences. When she was 8 years old, her mother discovered a shoebox under her bed with various microbes growing on agar plates. When confronted about the discovery, Nichole explained that she had swabbed her brother's throat (who had been sick the previous week) and was exposing the "germs" to different plant extracts in an effort to determine which would be most effective at eliminating the disease causing organisms. That was the start of her pursuit to become a biologist.

Nichole received both her Bachelors of Arts in Anthropology and Masters of Education in Curriculum & Instruction from the University of North Florida. She resides with her husband, Mark Mummaw, and their two basset hounds, Munsell Bishop-Mummaw and Mendel Bishop-Mummaw, in Jacksonville, FL.